1980

The production of a chromogenic cometabolite of m-chlorobenzoate by Pseudomonas fluorescens

Layne Mark Johnson
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THE PRODUCTION OF A CHROMOGENIC COMETABOLITE OF META-CHLOROBENZOATE BY PSEUDOMONAS FLUORESCENS

Iowa State University PH.D. 1980

The production of a chromogenic cometabolite of *m*-chlorobenzoate by *Pseudomonas fluorescens*

by

Layne Mark Johnson

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Ames, Iowa

1980

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QUOTATION

"To the ignorant, the great results alone are admirable, to the knowing, rather the infinite device and sleight of hand that made them possible."

Robert Louis Stevenson

LITERATURE REVIEW

Introduction

Hazardous wastes are being generated at an alarming rate and include such toxic chemicals as dichlorobenzene, cyanide, toluene, xylene, various chlorinated hydrocarbons and a wide variety of crop-protection chemicals. Aromatic compounds make up 46% of the total synthetic organic compounds produced in America and are of great importance as intermediates in commercial chemical syntheses because they are incorporated into thousands of speciality chemicals (Lu and Metcalf, 1975). In 1973, 1.5×10^7 pounds (6.8×10^6 kg) of benzoate were produced and although this compound represents no direct environmental danger, it is frequently used to construct chemicals hazardous to the environment (Lu and Metcalf, 1975).

The Environmental Protection Agency (EPA) has been charged with regulating hazardous wastes and currently 52 million tons (4.7×10^{10} kg) of toxic wastes fall within EPA jurisdiction (Smith, 1980a). But regulations alone, such as manufacturer identification, disposal site designation and transportation restrictions, do not directly solve the problem of toxic waste disposal.

Meanwhile, the U. S. State Department has disclosed plans which describe the transport and actual dumping of hazardous wastes overseas in third world countries, including Sierra Leone, Haiti, Chile, Liberia, Senegal and Nigeria (Smith, 1980b). Such practices have alarming technical, political and environmental ramifications. To transport wastes

to other unsuspecting countries for monetary recompense only relocates the toxic wastes and does nothing to initiate safe disposal. There are problems associated with waste disposal sites in the United States, and the EPA has recently requested the U. S. Justice Department to force eleven of the largest U. S. chemical corporations to clean up two existing sites (Des Moines Register and Tribune, 1980).

The cost of implementing EPA regulations for control and handling of toxic wastes has been estimated at a staggering $1 billion annually (Smith, 1980a). The EPA has focused most attention on the regulation of toxic wastes rather than on responsible disposal. David Costle, an EPA administrator, did not view the regulations as offering a final solution, but felt that they were "an essential first step" (Smith, 1980a) and the EPA is slated to designate disposal standards by October, 1980.

The magnitude of the problem that arises solely from chemical pesticide use can be appreciated by noting the quantity of chemicals used, produced or disposed of annually. In 1964, approximately 6.8×10^8 pounds (3.1×10^8 kg) of active pesticidal chemicals were manufactured (Alexander, 1969). By 1971, about 8.0×10^8 pounds (3.6×10^8 kg) of pesticides were applied to the environment (Munnecke, *et al.*, 1976). Approximately 1.2 billion pounds (5.4×10^8 kg) of pesticides were used in the United States during 1977 (USDA, 1977). It was projected that a 50% increase in the amount of pesticides that were used in 1976 will be seen by 1984 (Muhm, 1976) and growth rates of 6-8% per year are expected for the next several years (Doyle, 1975).

With each increase in the use of crop-protection chemicals and pro-

duction of other toxic chemicals there exists the severe problem of disposal. Reviews of pesticide disposal methods were presented by Day (1976) and Munnecke, *et al.* (1976). Characteristics for pesticide disposal systems, including cost, availability, convenience and effectiveness were described by Day (1976). These disposal methods include: (1) use as a diluent in spray tanks, (2) incineration at high temperature (1000° C), (3) soil injection by farm equipment, (4) photodecomposition by placement of the chemical on the soil surface or in a lagoon, (5) chemical degradation by chemical oxidation or reduction and alkaline or acid hydrolysis, (6) batch biodegradation under controlled microbial culture conditions, (7) disposal pits, such as several systems presently employed at Iowa State University, (8) special landfills and (9) storage (which only delays disposal). In addition to these types of disposal, Munnecke has developed a system utilizing immobilized enzymes for detoxification of 9 organophosphate pesticides (Munnecke, 1978).

Methods for disposal that are considered most unreliable are photodecomposition, chemical degradation and special landfills (Day, 1976). Although batch biodegradation is a slow, expensive and complex system, several investigators believe that biodegradation may be the single most important process for removing chemicals from the environment (Dagley, 1971) and several systems involving biodegradation have been presented (Munnecke, *et al.*, 1976).

Aromatic hydrocarbon degradation by microorganisms

Aside from the fact that several man-made compounds contain the aromatic nucleus, the ubiquity of aromatic rings is continually demonstrated because of their production by plant life. The most predominant form of naturally-occurring aromatic compounds is lignin, and other aromatic forms are found in aromatic amino acids and vitamins of every organism (Gottschalk, 1979). If the carbon in naturally-occurring compounds was not recycled, vast amounts would be locked up in stable benzene nuclei and taken out of circulation, thereby upsetting the general economy of nature (Dagley, 1971). Because many benzene derivatives prove recalcitrant to microbial degradation, they accumulate in the environment and pose a hazard to ecological integrity (Gibson, 1968). In order to circumvent such occurrences, it is necessary to understand aromatic degradation.

Degradation under aerobic conditions

Interest in microbial degradation of aromatic compounds by aerobic means has centered on (1) the study of metabolic intermediates, (2) the elucidation of the enzymatic mechanisms of ring hydroxylation and fission, and (3) the regulation of enzymes involved in aromatic metabolism (Gibson, 1968). Information has been gathered from studies conducted on several bacteria capable of growing at the expense of aromatic hydrocarbons, including members of the genera *Pseudomonas*, *Bacillus*, *Nocardia*, *Flavobacterium*, *Streptomyces* and *Rhodopseudomonas*.

It has been reported that most aromatic compounds, including benzoic acid, enter cells via a facilitated transport system (Cook and Fewson, 1972a and 1972b). After gaining entry into the cell, the aromatic nuclei are hydroxylated. The enzymes catalyzing the hydroxylation of the aromatic ring have been termed oxidases (Ornston and Parke, 1977), mixed-function oxidases (Evans, 1963), hydroxylases (Dagley, 1975a) or oxygenases referred to as oxygen transferases (Mason, 1955). Dihydroxylation of the aromatic ring is a prerequisite for ring fission and the hydroxyl groups may be situated *ortho* to each other, as in the cases of catechol or protocatechuic acid, or *meta* to each other, as in the cases of gentisate and homogentisate. These arrangements of hydroxyl groups are probably required to mediate shifts of electrons that occur during ring fission reactions (Dagley, 1975a). In cases where a benzene ring does not carry two hydroxyl groups, at least one additional hydroxyl group must be attached to an appropriate aromatic carbon in order for ring-opening to occur (Dagley, 1975b). The enzymes which mediate such hydroxylations are termed monooxygenases (or hydroxylases). Whether one or two hydroxyl groups are added to an aromatic ring is relatively unimportant, but that dihydroxylation is accomplished is of major concern to enzymes of aromatic ring fission. The hydroxylase enzymes serve to prepare the aromatic nucleus for ring fission by incorporating atomic oxygen into the substrate (Dagley, 1975a).

During the breakdown of phenylalanine and tyrosine, animals and bacteria form homogentisate as a key intermediate (Gottschalk, 1979). Homogentisate can be formed by *Pseudomonas acidovorans* following hydroxy-

lation of *m*-hydroxybenzoate, and the cleavage products, fumarate and pyruvate have been reported (Gottschalk, 1979).

As previously mentioned, *ortho* dihydroxylation of aromatic compounds results in the formation of the central "hub" intermediates protocatechuic acid and catechol; the former is a specific intermediate in the oxidation of *p*-hydroxybenzoic acid, and both may occur during benzoic acid oxidation. Catechol was first implicated in benzoate metabolism by Evans (1947). Stanier and Ornston (1973) summarized the catabolic routes that lead to catechol and protocatechuic acid, and some substrates metabolized via these two aromatic compounds are presented in Table 1.

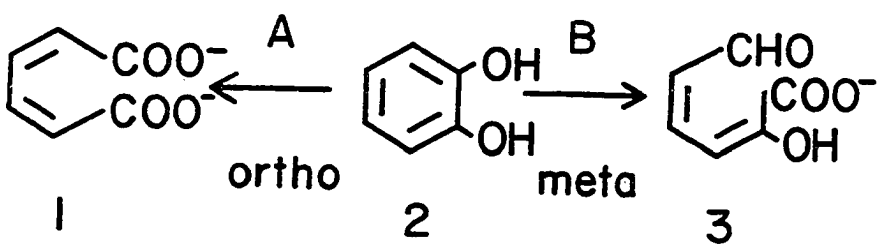
Two types of cleavage reactions exist for catechol and protocatechuic acid, and these include *ortho*-fission and *meta*-fission (Gibson, 1968, Gottschalk, 1979 and Dagley, 1971). The oxygenase enzymes which catalyze these ring-cleavage reactions are iron-sulfur proteins which incorporate both atoms of oxygen (O_2) into the substrate molecule (Figure 1). It should be understood that *ortho*- and *meta*-fission do not denote positioning of hydroxyl groups on aromatic rings (as discussed previously), but indicate specific degradation pathways utilized by microorganisms during aerobic aromatic dissimilation.

Since ring cleavage is a distinct biochemical step in aromatic degradation (Evans, 1963), substituted benzene nuclei allow microorganisms to choose, dependent upon their enzymatic constitution, a successful mode of attack (Gibson, 1968). Usually, bacteria catalyze hydroxylations to initiate one, and only one, pathway for complete degradation (Dagley, 1975b). Most aerobic bacteria that use aromatic

Table 1. Catechol and protocatechuic acid as central intermediates in the degradation of several aromatic compounds. (Gibson, 1968 and Gottschalk, 1979)

Compounds metabolized via	
catechol	protocatechuic acid
anthracene	*p*-aminobenzoate
anthranilate	benzoate
benzaldehyde	*m*-cresol
benzene	*p*-cresol
benzoate	5-dihydroxyshikimate
benzoyl formate	*m*-hydroxybenzoate
benzyl alcohol	*p*-hydroxybenzoate
o-cresol	*p*-hydroxybenzaldehyde
formyl kynurenine	*p*-hydroxybenzoyl formate
L-kynurenine	*p*-hydroxy-L-mandelate
mandelic acid	*p*-toluate
naphthalene	quinate
phenanthrene	shikimate
phenol	vanillate
salicylic acid	
toluene	
L-tryptophan	

Figure 1. The first steps in the *ortho*- or *meta*-cleavage of catechol (2). Two oxygenase enzymes may catalyze the ring fission of catechol. When *ortho*-fission occurs, which is by far the most predominant means of catechol dissimilation, *cis*, *cis*-muconate (1) is formed and can be completely mineralized via enzymes of the *B*-ketoadipic acid pathway. The production of *cis*,*cis*-muconate is catalyzed via catechol 1,2-oxygenase. On the other hand, *meta*-fission, which is a more rarely used mechanism, occurs when catechol is cleaved via catechol 2,3-oxygenase to yield 2-hydroxymuconic semialdehyde (3). Enzymes of the *meta*-fission sequence further dissimilate this compound to Kreb's cycle intermediates. (Dagley, 1975a)

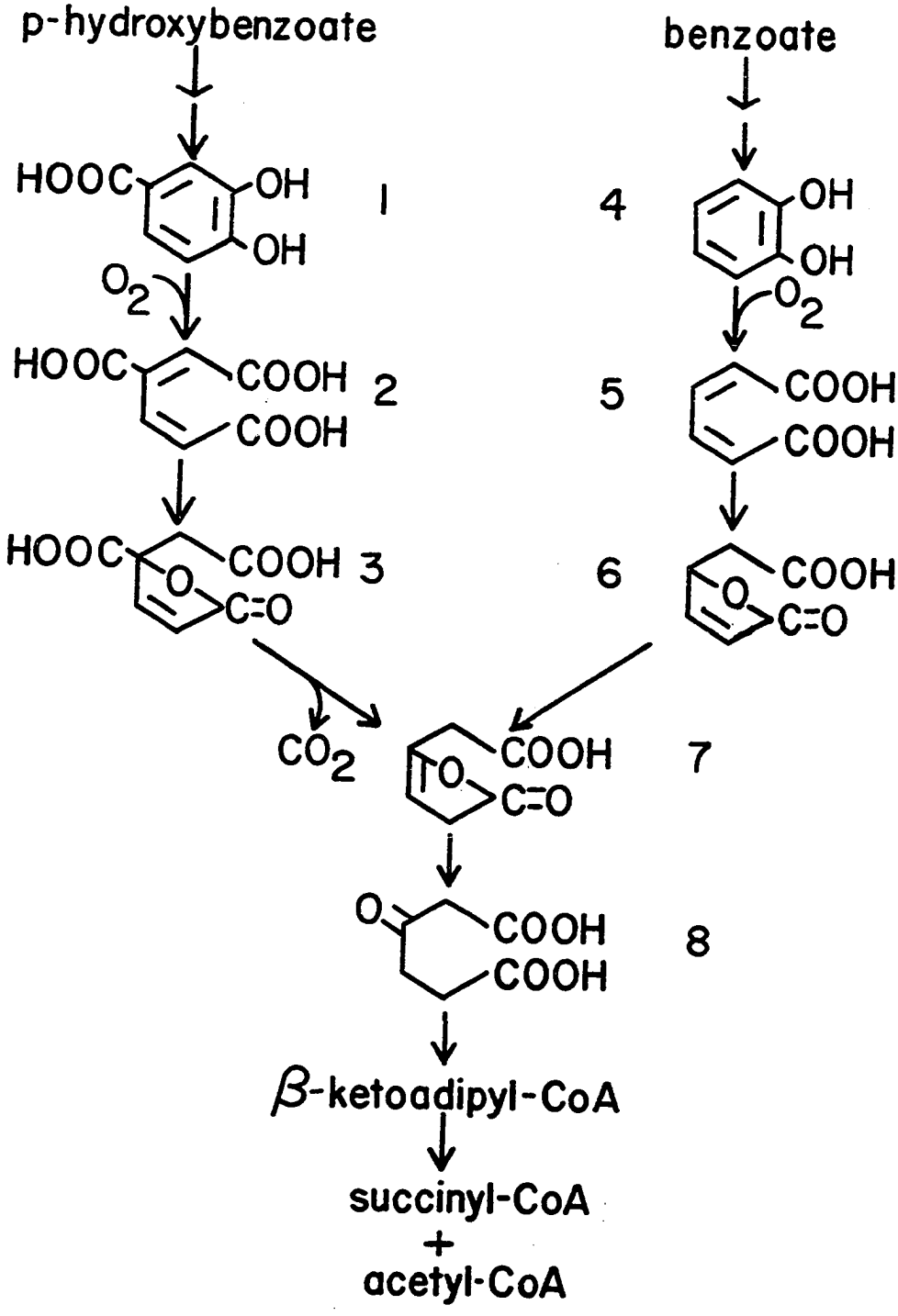

COO⁻
COO⁻
A
OH
OH
B
CHO
COO⁻
OH
ortho
meta
1
2
3

compounds as respiratory substrates attack them via one or another of two convergent branches of the *B*-ketoadipic acid (sometimes referred to as *B*-oxoadipic acid) pathway (Dagley and Nicholson, 1970, Gibson, *et al.*, 1968 and Stanier, 1976). An excellent review of the *B*-ketoadipic acid pathway has been furnished by Stanier and Ornston (1973).

Since an entire pathway is predestined once initiated, then one, and only one, branch of the *B*-ketoadipic acid pathway should be utilized during any given series of catabolic events included in this metabolic scheme. It has been recently observed, however, that separations of catabolic pathways leading to ring-fission substrates are often interconnectable and that the catabolism of aromatic compounds may be best represented by a "web" of interconnected sequences rather than a list of separate, distinct pathways (Crawford and Olson, 1978). Whatever the case, all enzymes in the sequences are inducible and are only present in small amounts when cells are grown in media such as nutrient broth or glucose plus basal salts (Dagley, 1975b). Enzymes for aromatic degradation are synthesized in much larger amounts when organisms are grown on aromatic substrates (Dagley, 1975b).

The conversion of catechol and protocatechuic acid to *B*-ketoadipate was reported by Ornston and Stanier (1966) and Ornston (1966a, 1966b and 1966c). Bacterial degradation of catechol and *p*-hydroxybenzoic acid via the *B*-ketoadipic acid pathway during *ortho*-fission metabolism is represented in Figure 2. The reactions of the two branches are chemically analogous and involve oxygenative cleavage, lactonization and endocyclic rearrangement of a double bond (Ornston and Parke, 1977).

Figure 2. The *B*-ketoadipic acid pathway. *Para*-hydroxybenzoate and benzoate may be degraded after respective conversion to either protocatechuic acid (1) or catechol (4). Protocatechuic acid (1) may be enzymatically cleaved by protocatechuate 3,4-oxygenase to yield *B*-carboxy-*cis*,*cis*-muconate(2). This compound is converted to *gamma*-carboxymuconolactone (3) by the action of *B*-carboxymuconate lactonizing enzyme. *Beta*-ketoadipate enol-lacton (7) is formed from *gamma*-carboxymuconolactone after decarboxylation which is catalyzed by the action of *gamma*-carboxymuconolactone decarboxylase. Catechol (4) undergoes *ortho*-fission initially after being converted to *cis*,*cis*-muconate (5). This compound is then converted by the muconate lactonizing enzyme. After (+)muconolactone (6) is formed, an isomerization reaction takes place which is mediated by muconolactone isomerase, to yield *B*-ketoadipate enol-lactone (7). As stated previously, this is also an intermediate formed during the *ortho*-fission of *p*-hydroxybenzoate and protocatechuic acid (1), and therefore represents a point of convergence during protocatechuic acid (1) or catechol (4) degradation. *B*-ketoadipate enol-lactone undergoes a hydrolase-mediated reaction to form *B*-ketoadipic acid (8). The action of specific transferases results in the conversion of *B*-ketoadipic acid to *B*-ketoadipyl CoA which ultimately is converted to succinyl CoA and acetyl CoA. (Dagley, 1975a, Stanier and Ornston, 1966, Evans, 1963 and Feist and Hegeman, 1969)

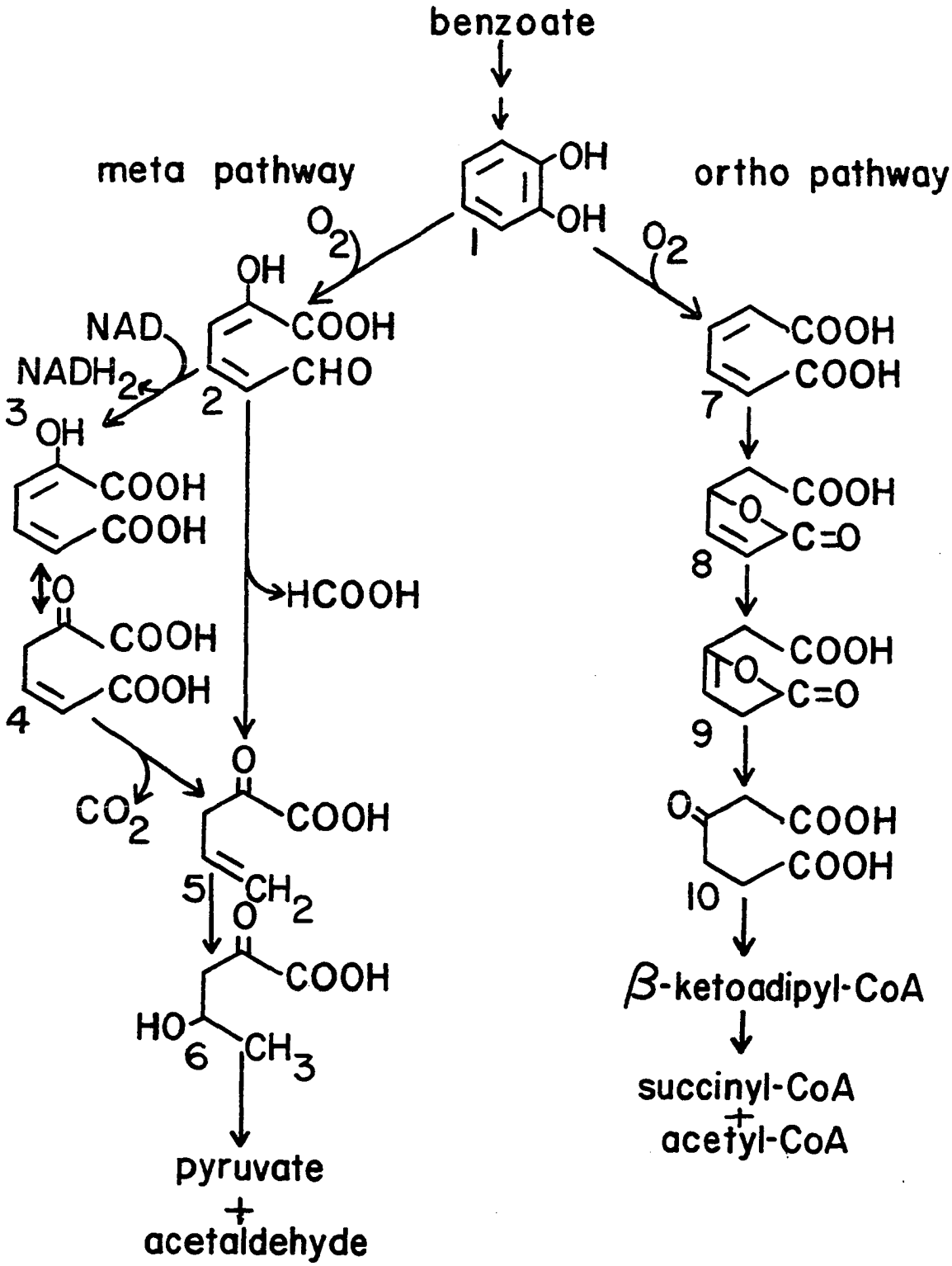

p-hydroxybenzoate
benzoate
HOOC
OH
OH
1
4
O_2
HOOC
COOH
COOH
2
5
HOOC
COOH
O
C=O
3
6
COOH
O
C=O
CO_2
COOH
O
C=O
7
O
COOH
COOH
8
β-ketoadipyl-CoA
succinyl-CoA
+
acetyl-CoA

Another mechanism of aromatic ring cleavage occurs when *o*-dihydroxyphenols (e.g. catechol) are cleaved via *meta*-fission pathways. This route of dissimilation of catechol is NAD-dependent (Stanier and Ornston, 1973). The *meta*- and *ortho*-fission of catechol are presented in Figure 3. The primary physiological benefit of the *meta*-fission pathway is that it allows the degradation of acids bearing an alkyl substituent on the aromatic ring (Feist and Hegeman, 1969).

The aromatic ring-cleavage enzymes of *o*-dihydroxyphenol degradation via *ortho*- or *meta*-fission have been examined and summarized by Dagley, Evans and Ribbons (1960), Evans (1963), Ornston and Parke (1977) and Stanier and Ornston (1973). During *ortho*-cleavage of either catechol or protocatechuic acid, pyrocatechase (catechol 1,2-oxygenase) and protocatechuic acid oxidase (protocatechuate 3,4-oxygenase) break the carbon-carbon bonds between two adjacent hydroxyl groups. The resulting ring fission product, *cis,cis*-muconate (in the case of catechol degradation) is subsequently lactonized by a Mn^{2+}-dependent enzyme referred to as muconate lactonizing enzyme. A delactonating enzyme hydrolytically cleaves the muconolactone to yield *B*-ketoadipic acid. The bond between carbon numbers two and four is then cleaved in the presence of coenzyme A and the substrates succinate and acetyl CoA are ready for use in the tricarboxylic acid cycle (Figure 3).

During *meta*-fission, catechol 2,3-oxygenase (*meta*-pyrocatechase) or protocatechuate 4,5-oxygenase may cleave the carbon-carbon bond adjacent to a hydroxyl group in catechol. The formation of 2-hydroxymuconic semialdehyde subsequently induces 2-hydroxymuconic

Figure 3. The *ortho*- and *meta*-fission of benzoate. The *ortho*-fission pathway mechanisms have been presented in Figure 4, but the intermediates catechol (1) *cis,cis*-muconate (7), (+)muconolactone (8), *B*-ketoadipate enol-lactone (9) and *B*-ketoadipate (10) are also presented here for comparison. During *meta*-fission of catechol (1), catechol 2,3-oxygenase catalyzes the production of 2-hydroxymuconic semialdehyde (2) which is dehydrogenated to form 4-oxalocrotonate (which may exist in the enol (3) or keto (4) forms). 2-oxopent-4-enoate (5) may be formed either directly from 2-hydroxymuconic semialdehyde (2) following a hydrolase reaction which also results in the formation of formic acid or via 4-oxalocrotonate (enol, 4) following decarboxylation. Whatever the mode of formation, 2-oxopent-4-enoate (5) undergoes a hydratase reaction to yield 4-hydroxy-2-oxovalerate (6). This compound is then cleaved by a specific aldolase to form pyruvate and acetaldehyde. (Williams and Murray, 1974)


benzoate
meta pathway
ortho pathway
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OH
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O_2
O_2
OH
COOH
CHO
2
NAD
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COOH
COOH
HCOOH
O
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COOH
4
CO_2
O
COOH
5
CH_2
O
COOH
HO
6
CH_3
pyruvate
+
acetaldehyde
COOH
COOH
7
COOH
O
C=O
8
COOH
O
C=O
9
O
COOH
COOH
10
β-ketoadipyl-CoA
succinyl-CoA
+
acetyl-CoA

semialdehyde dehydrogenase to result in the production of 4-oxalocrotonate (enol). The enol form of this compound may then tautomerize to the keto form, which may then be decarboxylated by the action of 4-oxalocrotonate decarboxylase to form 2-oxopent-4-enoate. The direct production of 2-oxopent-4-enoate from 2-hydroxymuconic semialdehyde may be mediated via the action of 2-hydroxymuconic semialdehyde hydrolase with the concomitant production of formic acid.

Once formed, 2-oxopent-4-enoate is converted to 4-hydroxy-2-oxovalerate, which is eventually cleaved by a specific aldolase to yield pyruvate and acetaldehyde. Although it is generally considered that benzoate is dissimilated strictly via *ortho*-fission mechanisms (Feist and Hegeman, 1969), it has been shown that *Pseudomonas putida (arvilla)* mt-2 may cleave benzoate via the *meta*-fission pathway when the strain carried the TOL plasmid (Williams and Murray, 1974). If strains of *P. putida (arvilla)* mt-2 had lost the TOL plasmid, only the *ortho*-sequence (*B*-ketoadipic acid pathway) was used by the organism (Williams and Murray, 1974).

Ornston and Parke (1977) have reviewed the aspects of regulation of the *B*-ketoadipic acid pathway. It may be recalled from Figures 2 and 3 that catechol is an intermediate formed during the catabolism of benzoate. Most all representatives of the genus *Pseudomonas* that are fluorescent utilize inducible enzymes of the *B*-ketoadipic acid pathway to degrade aromatic compounds (Ornston and Parke, 1977), but when catechol is degraded via *ortho*-fission during steps of the *B*-ketoadipic acid pathway, it does not induce the enzymes of the sequence

(Gottschalk, 1979). Alternatively, product induction occurs twice after exposing non-induced cells to catechol.

The first example of product induction is observed when *cis,cis*-muconate triggers the production of catechol 1,2-oxygenase, which is typically formed at extremely low levels in the absence of inducer (Ornston and Parke, 1977). *Cis,cis*-muconate plays a dual role in the *B*-ketoadipic acid pathway; it not only serves as a product inducer for the production of the cleavage enzyme, catechol 1,2-oxygenase, but it also serves as a substrate inducer when it coordinately induces both muconate lactonizing enzyme and muconolactone isomerase. After being rearranged by these two enzymes, *cis,cis*-muconate appears as *B*-ketoadipate enol-lactone. This compound does not induce *B*-ketoadipate enol-lactone hydrolase (the enzyme responsible for *B*-ketoadipic acid formation); it must be converted to *B*-ketoadipate in order for product induction of *B*-ketoadipic enol-lactone hydrolase to occur.

The most remarkable feature of the induction pattern observed during degradation is that strains of fluorescent pseudomonads growing on substrates that are degraded via catechol also gratuitously synthesize two enzymes (carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase) that are uniquely associated with the protocatechuic acid branch of the degradation sequence. Such substrate induction, via *cis,cis*-muconate, exemplifies the very close relatedness of the catechol and protocatechuic acid branches of the *B*-ketoadipic acid pathway. Feist and Hegeman (1969) also reported that gratuitously-synthesized enzymes of *meta*-cleavage pathways could be

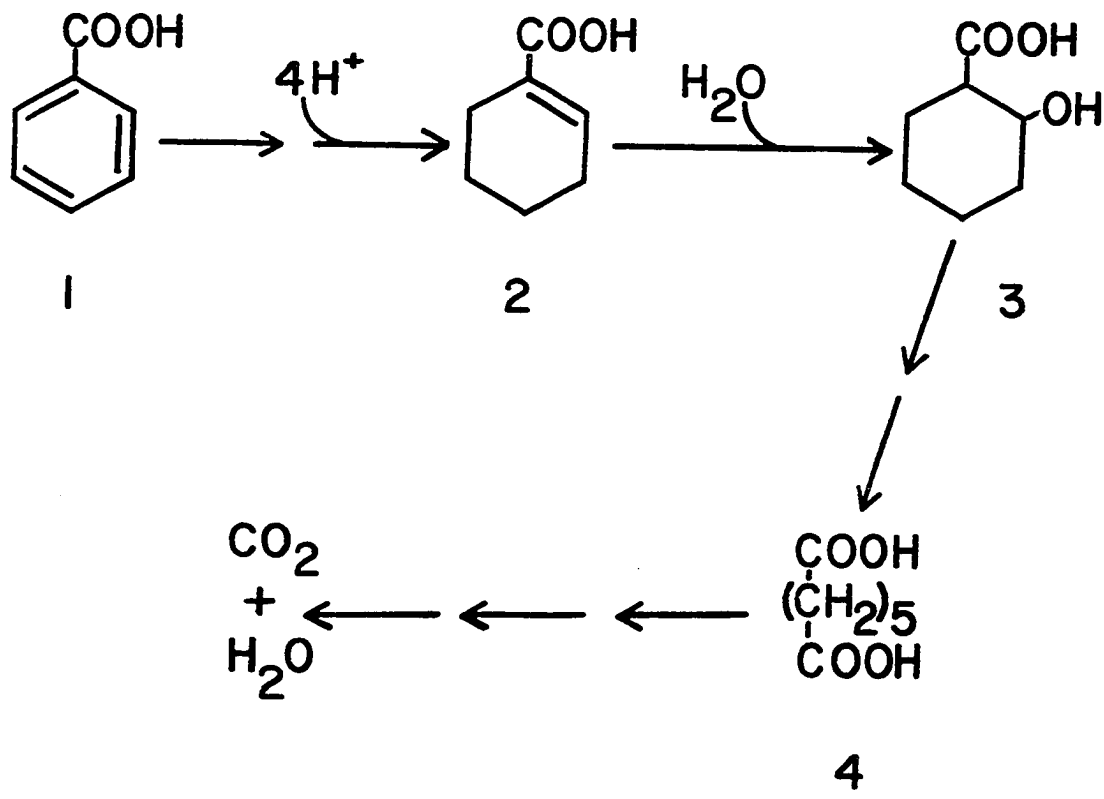
induced in *Pseudomonas putida* when non-metabolizable inducers are employed.

Degradation under anaerobic conditions

Compared to aerobic degradation of aromatic compounds, breakdown under anaerobic conditions has received comparatively little consideration. One unique reductive pathway has been reported for *Rhodospeudomonas palustris* (Dutton and Evans, 1969). This Gram-negative, purple non-sulfur bacterium has the ability to photoassimilate aromatic compounds anaerobically in light, but is unable to use aromatic carbon sources as respiratory substrates (Stanier, 1976). A reductive pathway utilized during the initial steps of benzoate photometabolism by *R. palustris* demonstrates the conversion of benzoate to a saturated dicarboxylic acid, pimelate (Figure 4). Under anaerobic conditions, cultures of *R. palustris* grow well on benzoate and *m*-hydroxybenzoate, but are unable to use *o*-hydroxybenzoate, protocatechuate or nicotinate (Dutton and Evans, 1969). The enzymes which catalyze these reactions are extremely oxygen-sensitive, and the photoassimilation of benzoate is arrested when cells are exposed even to traces of oxygen (Stanier, 1976). Under aerobic conditions, oxidative enzymes allow *R. palustris* to grow rapidly on *p*-hydroxybenzoate and protocatechuate, but prohibit the organism from utilizing either benzoate, *o*- and *m*-hydroxybenzoate or catechol as growth substrates (Dutton and Evans, 1969).

Additional evidence has been presented for aromatic degradation

Figure 4. Proposed pathway for the photometabolism of benzoate by *Rhodopseudomonas palustris*. During this anaerobic degradative pathway, benzoate (1) is first reduced, producing an aliphatic cyclic acid, cyclohex-1-ene-1-carboxylate (2). Hydration produces 2-hydroxycyclohexane carboxylate (3) which is converted to the ring cleavage product, pimelic acid (4). (Dutton and Evans, 1969)


COOH
4H+
COOH
H2O
COOH
OH
1
2
3
COOH
(CH2)5
COOH
CO2
+
H2O
4

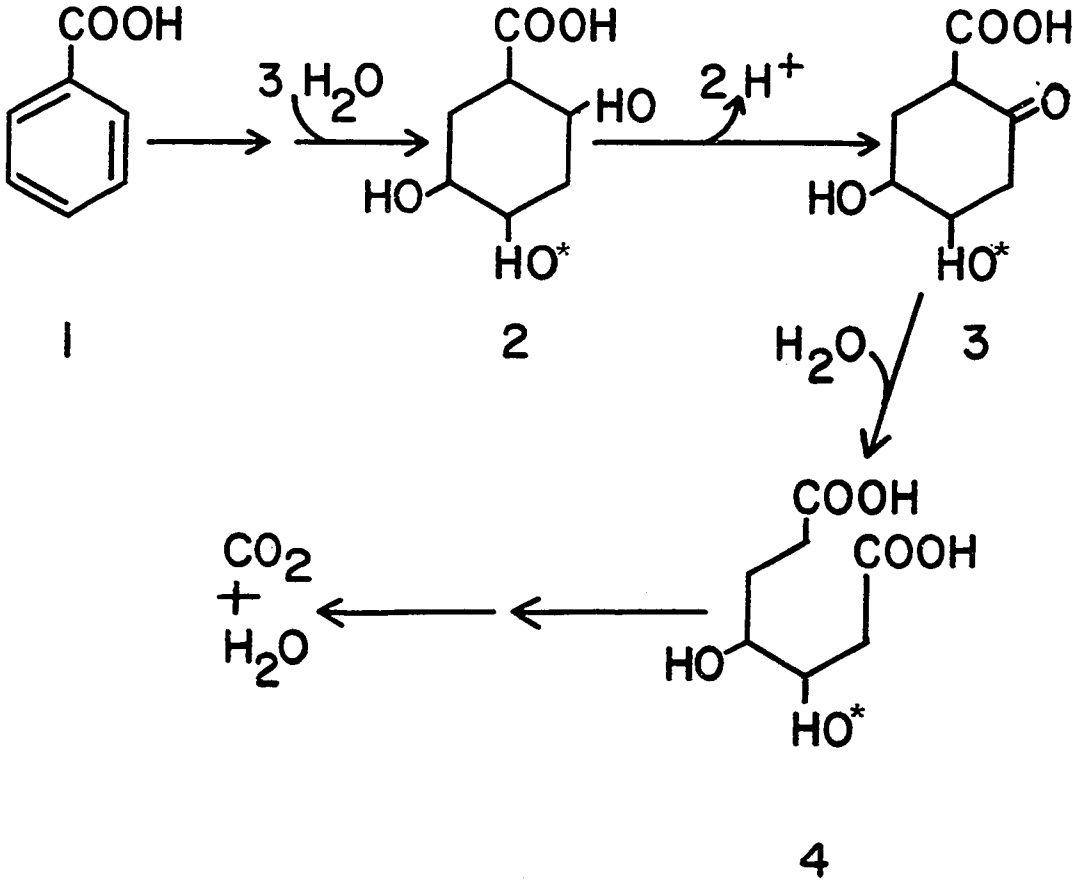
under anaerobic conditions during nitrate respiration (Taylor, *et al.*, 1970 and Williams and Evans, 1975). Degradation mechanisms for the anaerobic metabolism of the benzene nucleus during nitrate respiration by *Pseudomonas* PN-1 were discussed by Taylor, *et al.* (1970). These investigators concluded that ring fission was mediated by a mechanism that involved the addition of water to the aromatic ring (Figure 5).

The degradation of aromatic nuclei under anaerobic conditions during methane fermentation has been presented by Ferry and Wolfe (1976) and Healy and Young (1978). When catechol and phenol were degraded by a methanogenic population of bacteria, metabolism proceeded via reduced intermediates and stoichiometric amounts of methane and carbon dioxide were produced under completely anaerobic conditions (Healy and Young, 1978). This work indicated that aromatic ring compounds were not refractory under strictly anaerobic conditions and that heterotrophic bacteria possess the enzymes involved in reductive pathways.

Substituent effects on aromatic hydrocarbon degradation

Attention continued to be given to the reactions of aromatic degradation some twenty-five years after concentrated studies began. Although the ellucidation of several pathways had been fully accomplished by the mid- to late 1960s, several groups of investigators began studying various subtituents attached to the benzene nucleus and the effects these substituents had on biodegradation. Knowledge of the enzymatic breakdown of halogenated benzene nuclei had given much insight into the problem of molecular recalcitrance and microbial

Figure 5. Hypothetical scheme for the anaerobic metabolism of benzoate during nitrate respiration by *Pseudomonas* PN-1. After benzoate (1) is hydrated to form trihydroxycyclohexane carboxylic acid (2), it has been indicated that a oxidative step occurs and dihydroxycyclohexan-2-one-1-carboxylic acid (3) is formed. A molecule of water is then incorporated in the pathway and dihydroxypimelic acid (4) is formed. Asterisks indicate that hydroxyl groups have been arbitrarily assigned, and the groups may also be attached to carbon numbers 3 or 5 on the aromatic ring. (Taylor, *et al.*, 1970)


COOH
3 H2O
HO
HO
HO*
2 H+
O
1
2
H2O
3
4
CO2
+
H2O

fallibility (Alexander, 1965). The environmental impact of such studies is still speculative, but it has been indicated that our present knowledge of aromatic catabolism would have allowed man to predict without reservation, that DDT would be resistant to microbial attack (Dagley, 1971). The unrestricted use of this compound might have been avoided.

As environmental awareness increased during the mid-1960s and especially during the 1970s, reports of the effects of benzene substituents on degradative processes began to appear in the literature (Alexander, 1965). Alexander and Lustigman (1966) reported that a mixed population of bacteria from soil degraded substituted benzenes at different rates. These investigators observed that chloro-, sulfonate and nitro- groups retarded the rate of microbiologically-mediated degradation, whereas carboxyl and phenolic hydroxyl groups favored attack by soil organisms. Although Alexander and Lustigman (1966) reported that *meta* isomers of the compounds studied typically were most resistant to attack, they also observed that *ortho* isomers of toluic acid, nitrophenol and toluidine were most resistant. The rate and degree of attack reported by these authors most probably reflected the degradative capacities of the specific populations studied.

A trend in the literature discussing substituent effects on degradation is quite difficult to detect. In the case of cresols, hydroxybenzoates, fluorobenzoates, chlorophenoxyacetates and methoxybenzoates, it has generally been considered that the *para* derivative of a compound is more readily used than *ortho* or *meta* derivatives (MacRae and Alexander, 1965). It has been reported that soil sus-

pensions never rapidly cleaved rings containing a halogen in a *meta* position (MacRae and Alexander, 1965).

During a study of benzoate oxidases prepared from *Micrococcus ureae* Et, *Pseudomonas aeruginosa* B-23 and *Pseudomonas fluorescens* Mb-15, Ichihara, *et al.* (1962) reported that of the various benzoate derivatives investigated, those halogenated at the *meta* position could be oxidized to halogenated catechols. These investigators indicated that the benzoate oxidase enzyme exhibited wide substrate specificities but that hydroxylation of halogenobenzoates was favored when the halogen substituent was attached at the *meta* position.

Hughes (1965) reported that washed suspensions of *Pseudomonas fluorescens* grown with benzoate as a sole carbon source, oxidized mono-halogenobenzoates. The descending order of effectiveness for these oxidations were: benzoate, fluorobenzoates, chlorobenzoates, bromobenzoates and iodobenzoates. In this study, halogenobenzoates were not used as sole carbon sources for growth, and the compounds did not increase growth when *P. fluorescens* was grown on succinate. Similar findings were reported by Ichihara, *et al.* (1962) when they found that benzoate oxidase was not induced when bacteria were grown in a medium containing citrate as a main source of carbon. Hughes (1965) felt that the inability of *P. fluorescens* to use any of the halogenated benzoates for growth was due to the inability of the bacterium to liberate the halogen and carry the oxidation to a stage where carbon could be assimilated. This investigator also indicated that halogenobenzoates may inhibit the induction of oxidizing enzymes.

Ichihara, *et al.* (1962) and Hughes (1965) were studying a series of events which may now be considered as cometabolism, yet no mention of this phenomenon was made in either of their publications. During the early 1960s cometabolism was in its infancy, but was rapidly maturing to the point where its significance in the biodegradation of man-made compounds was to be realized.

A link between halogenated aromatic degradation and cometabolism (cooxidation) appeared in the literature in 1968 when Gibson and co-workers indicated that the cooxidative phenomenon may be of use in their studies (Gibson, *et al.*, 1968). These investigators reported that *Pseudomonas putida*, when grown with toluene as a sole source of carbon, oxidized chloro-, bromo-, iodo-, and fluorobenzoates to their respective catechol derivatives. No discussion of cooxidative processes was included in this 1968 publication, although. The existence of such mechanisms in the work can be seen in retrospect.

Cometabolism

The origin of cometabolism can be traced to work done by E. R. Leadbetter and J. W. Foster which appeared in 1959 (Leadbetter and Foster, 1959). These workers found that *Pseudomonas methanica*, an obligate methane-utilizing bacterium, oxidized several gaseous paraffinic hydrocarbons to oxidation products which contained corresponding numbers of carbon atoms. The bacterium was cultivated in broth media under a gas phase consisting of air and the growth substrate, methane. Media were supplemented with ethane, propane or butane. From media

supplemented with ethane, the workers isolated the oxidized products acetic acid and acetaldehyde; from propane-supplemented media, propionic acid and acetone were isolated. *n*-Butyric acid and 2-butanone were isolated from media supplemented with *n*-butane. These remarkable processes were recognized because *P. methanica*, which is capable of using only methane as a growth substrate, oxidized several non-utilizable carbon sources. Initially, cooxidation was considered a process by which a microorganism could oxidize a substance but could use neither carbon nor energy from the oxidation.

In 1963, Jensen suggested that the more general term, cometabolism be used to describe cooxidative processes (Jensen, 1963). This term was used not only to describe previously defined processes, but was expanded to include dehalogenation reactions.

Cooxidation and cometabolism have been used interchangeably so frequently in the literature that the two terms are often considered to be synonymous (Horvath, 1972). An excellent review of cometabolism was presented by Horvath in 1972, and in this paper the phenomenon was described in terms of organic pollutants (Horvath, 1972).

In 1979, Perry presented a review of microbial cooxidations in which he defined cooxidation as a technique (Perry, 1979) and supported his definition in accordance with the definition established by J. W. Foster (Leadbetter and Foster, 1959 and 1960). Foster (1962) defined cooxidation as follows: "Non-growth hydrocarbons are oxidized when present as cosubstrates in a medium in which one or more different hydrocarbons are furnished for growth". It was also stated by Foster (1962)

that the inability to grow at the expense of a substrate (due to molecular recalcitrance) was not always the result of an organism's inability to attack the substrate, but rather in its inability to assimilate the products of oxidation. The work described by Foster (1962) and by Leadbetter and Foster (1959 and 1960) involved cooxidations of paraffinic hydrocarbons, cycloparaffins and aromatic compounds, whereas Horvath (1972) applied the term cometabolism to describe the conversion of pesticides and other organic pollutants that were typically resistant to microbial attack.

In contrast to the cooxidative technique described by Perry (1979), Horvath extended the process to include the oxidation of non-utilizable substrates by resting cell suspensions previously grown on substances capable of supporting microbial growth (Horvath, 1972). Therefore, cometabolism did not infer the presence or absence of the growth substrate during an oxidation. Perry (1979) recoiled at such use of the term and felt that the use of either cometabolism or cooxidation to describe conversion of non-growth substrates by resting (non-proliferating) cells was completely inappropriate. Perry (1979) felt that the enzymatic conversion of a substrate via non-proliferating cell suspensions at the expense of an enzyme of broad specifity, might be best described as bioconversion. This author thought that no "co-" (with, together, joint) was involved when resting cells converted non-growth substrates. D. T. Gibson also believed that a cometabolic process might best be described by recognizing that several micro-organisms merely possess "broad spectrum enzymes" (personal communication,

July, 1979, University of Texas, Austin).

In his 1972 review, Horvath felt that a survey of the literature did not reveal a precise definition of cometabolism. Although some disagreement concerning the definition of cometabolism has existed in the literature, a working definition can be established. Since the work reported in this dissertation involves the cometabolism of an organic pollutant closely related to some chlorinated aromatic herbicides, the definition of cometabolism proposed by Horvath (1972) is most suitable.

Cometabolism can be defined as the ability of a microorganism to affect a change upon a substance without utilization of any carbon, some other nutrient element, or energy derived from the change to support microbial growth. The organisms involved with the particular cometabolic process contain enzymes capable of modifying the substrate without producing ATP or generating products that fit into biosynthetic pathways (Alexander, 1980). Cometabolism can describe the oxidation of non-utilizable substrates by resting cell suspensions previously grown at the expense of substances capable of supporting microbial growth. Also, Horvath (1972) stated that the proof of disappearance of substrate and accumulation of end products is necessary to clearly demonstrate cometabolism; however, the duration of the existence of accumulated end products has never been specified.

Besides using cometabolism as a technique to degrade organic compounds in nature, other biochemical techniques utilizing the phenomenon have been developed and were summarized by Horvath (1972).

Cometabolism has been used to demonstrate the accumulation of a wide variety of halogen-substituted metabolites (Horvath and Alexander, 1970a). Cometabolism has also been used in the aromatization of steroids into equilin by *Nocardia rubra* (Sehgal and Vezina, 1970). Mitruka and Alexander (1969) presented evidence that cometabolism could be used as an adjunct to gas-chromatographic procedures designed for the detection of low bacterial cell densities. Furthermore, cometabolism has been used to develop spectrophotometric assays (Ribbons and Senior, 1970) and may also be used to trace metabolic pathways (Horvath and Alexander, 1970a).

Aside from the fact that cometabolism has been supported in the literature by several groups of investigators, it has also been criticized. Hulbert and Kraweic (1977) offered criticism when noting that the descriptive features of cometabolism did not distinguish it from ordinary metabolism. Yet, it is presently considered that cometabolism contrasts markedly with typical growth-enhancing degradative sequences because population sizes do not increase with time during cometabolic transformations and products of typical metabolism either do not accumulate or persist only for short periods of time (Alexander, 1980).

Hulbert and Kraweic (1977) never assembled a precise definition of cometabolism, but believed that cometabolism failed to describe a biologically meaningful, previously unrecognized distinct and general condition. In effect, Hulbert and Kraweic glibly stated that the distinctive feature of cometabolism appeared to be that "its observers

were surprised by the degradative potential of microbes" (Hulbert and Kraweic, 1977). These authors also criticized cometabolism on the basis that degradation of cometabolites in the absence of other substrates had been reported (Horvath, 1972 and Horvath and Flathman, 1976). Hulbert and Kraweic concluded that cometabolism did not differ from catabolism or anabolism and that cometabolism "is not an intrinsic and distinct activity of an organism, but is an expression of the bias of an experimenter; the transformation of a material is termed 'cometabolism' because the investigator did not expect the material to be metabolized". Continued use of the term, it was felt, could only lead to serious misconception concerning the immediate capacity of microorganisms to rid the environment of the noxious materials, and therefore the use of the word should be abandoned.

Prior to such criticism, Horvath (1972) had stated that co-metabolism of environmentally important compounds dictated a re-examination of the concept of molecular recalcitrance (Alexander, 1965). The term recalcitrant had been used to describe organic pollutants that resisted change because of the fallibility of microorganisms (Alexander, 1967b). Alexander (1965) defined molecular recalcitrance as "a stubbornness on the part of certain molecules to succumb to microbiological attack" and microbial fallibility as lack of decomposition "due to inadequate catabolic potentials on the part of the microorganisms involved". But microbial fallibility was found to occur, for example, when naturally occurring bacterial spores, humic acid and fungi resisted degradation for years (Alexander, 1967a). Results

obtained with cometabolism indicated that the concept of molecular recalcitrance may become obsolete because compounds previously designated as recalcitrant were later shown to be subject to cometabolism (Horvath, 1972).

Recently, Alexander, (1980) summarized the environmental consequences of cometabolism which included: (1) the population of a specific microorganism does not increase because of the introduction of a non-utilizable substrate that may be subject to cometabolism; (2) the rate of cometabolic transformation will not increase with time because the active organism does not increase as a result of the introduction of the non-utilizable substrate; and (3) an organic product of the reaction will accumulate because the active organism cannot completely mineralize (convert to carbon dioxide and water) the original substrate. Because the extent of cometabolic transformation is not great, the product may endure until a population can metabolize or cometabolize it further, or until non-biological removal occurs. Also, enrichment culture techniques that are typically used to isolate specific microorganisms cannot be used successfully to isolate organisms capable of undertaking cometabolic processes. This is due to the fact that the organisms cannot obtain carbon, energy or other required nutrients from the cometabolizable substrate.

During the 1970s, several papers appeared in the literature that described cometabolism of halogenated benzoates and halogenated catechols. Horvath (1970) presented evidence that an *Achromobacter* sp. possessed a *meta*-cleaving enzyme, catechol 1,6-oxygenase. This

organism had the capacity to cometabolize 3-methylcatechol, 4-chlorocatechol and 3,5-dichlorocatechol to their respective 2-hydroxymuconic semialdehyde derivatives. These products accumulated in media and their production was significant because *meta*-cleavage of halogenated catechols had not been reported prior to this work. Concomitantly, Horvath and Alexander (1970a) communicated that the phenomenon of cometabolism may be exploited for the production of microbial products in relatively large yields. Cometabolism showed great promise as a means of producing large quantities of biochemicals for the fermentation industry and for tracing metabolic sequences.

Several papers initially appeared in the literature during the early 1970s and presented evidence for the cometabolism of *m*-chlorobenzoate (3-chlorobenzoate) and other chlorobenzoates by several different genera (Horvath, 1973, Horvath and Alexander, 1970b, Horvath, *et al.*, 1975, Spokes and Walker, 1974 and Walker and Harris, 1970). Horvath and Alexander (1970b) isolated an *Arthrobacter* sp. that cometabolized *m*-chlorobenzoate to an accumulated product identified as 4-chlorocatechol when the organism was grown at the expense of sodium benzoate. This product was identified via thin-layer chromatography, ultraviolet and infrared spectroscopy.

Two *Azotobacter* species, including *A. vinelandii* Lipman (NCIB 8660) and *A. chroococcum* were isolated from soil and could use sodium benzoate as a source of carbon and energy (Walker and Harris, 1970). Growth was not evident when organisms were placed in a medium of basal salts containing either 2-(*o*-), 3-(*m*-) or 4-(*p*-)chlorobenzoate. As

would be expected, these authors reported that benzoate or chlorobenzoate oxidation was not caused by constituitive enzyme systems and both organisms formed 3-chlorocatechol from 3-chlorobenzoate by cometabolic processes. The product was identified via gas-liquid chromatography and ultraviolet spectroscopy. These results indicate that *meta*-substituted benzoate was more easily oxidized than *ortho*- or *para*-substituted benzoates and correspond to results previously obtained by Ichihara, *et al*. (1962).

The substantiation of chlorobenzoic acid cometabolism by different genera of soil bacteria was also presented by Spokes and Walker (1974). The cometabolism of mono-chlorobenzoates was examined in studies of four *Pseudomonas* strains, an *Achromobacter* sp., three *Nocardia* strains, *Mycobacterium coeliacum* and a *Bacillus* sp. Each organism was grown on benzoic acid as a sole carbon source. Benzoate-grown cells of three of the pseudomonads and three *Nocardia* spp. oxidized only *m*-chlorobenzoate. 4-Chlorocatechol accumulated and was identified, via color production in the presence of ferric chloride ($FeCl_3$) and melting point determinations. The possibility of 3-chlorocatechol production during the cometabolic process was also noted.

Horvath (1973) reported that cometabolism of chlorobenzoates could be enhanced by employing the co-substrate enrichment technique. This study indicated that chlorobenzoate degradation by bacteria contained in an activated sludge inoculum could be enhanced when organisms used glucose as a co-substrate. Although it had previously been shown that readily utilizable substrates did not enhance aromatic

degradation (Ichihara, *et al.*, 1962 and Hughes, 1965), Horvath (1973) felt that the co-substrate, glucose, primarily served to increase the numbers of organisms capable of effecting an oxidation of halogenated material, rather than inducing a specific population or set of enzymes within a population. In another study of cometabolism by natural microbial populations (Horvath, *et al.*, 1975), *m*-chlorobenzoate was initially attacked via a cometabolic mechanism and was further degraded to inorganic chloride and organic intermediates that could support microbial growth. Again, 3-chlorocatechol was identified as an initial accumulated product, and its ultimate disappearance was attributed to the actions of a changing population.

Biodehalogenation

Although the details of dehalogenation were never examined in great depth during cometabolism studies, some insights of the mechanisms of dehalogenation have been obtained. Numerous biodehalogenation mechanisms have been summarized by Castro (1977); microbiologically-mediated mechanisms can include nucleophilic substitution (where an external hydroxyl group replaces a halogen) or reductive dehalogenation, as exemplified in the conversion of DDT to DDD.

The dehalogenation of aliphatic acids by several *Pseudomonas* spp. and a *Nocardia* sp. has been described by Castro and Bartnicki (1965) and Hirsch and Alexander (1960). Castro and Bartnicki (1968) have also reported the conversion of 2,3-dibromopropanol into glycerol by a partially purified halohydrin epoxidase isolated from a species

of *Flavobacterium*.

Two halidohydrolases, isolated from a soil pseudomonad, were described by Goldman, *et al*. (1968). These enzymes were induced by either chloroacetate or dichloroacetate, exhibited pH optima of 9.0 to 9.4 and acted via a nucleophilic substitution mechanism.

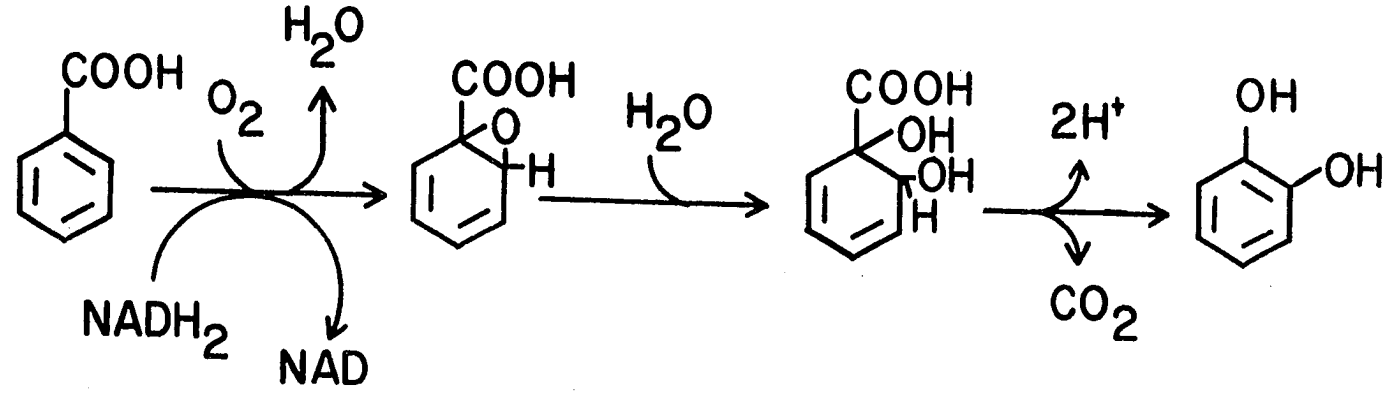
Aromatic carbon-halogen bond cleavage has been recently described by Janke and Fritsche (1978 and 1979). These authors have presented data which substantiate the thesis that microorganisms dehalogenate a variety of aliphatic and aromatic hydrocarbons.

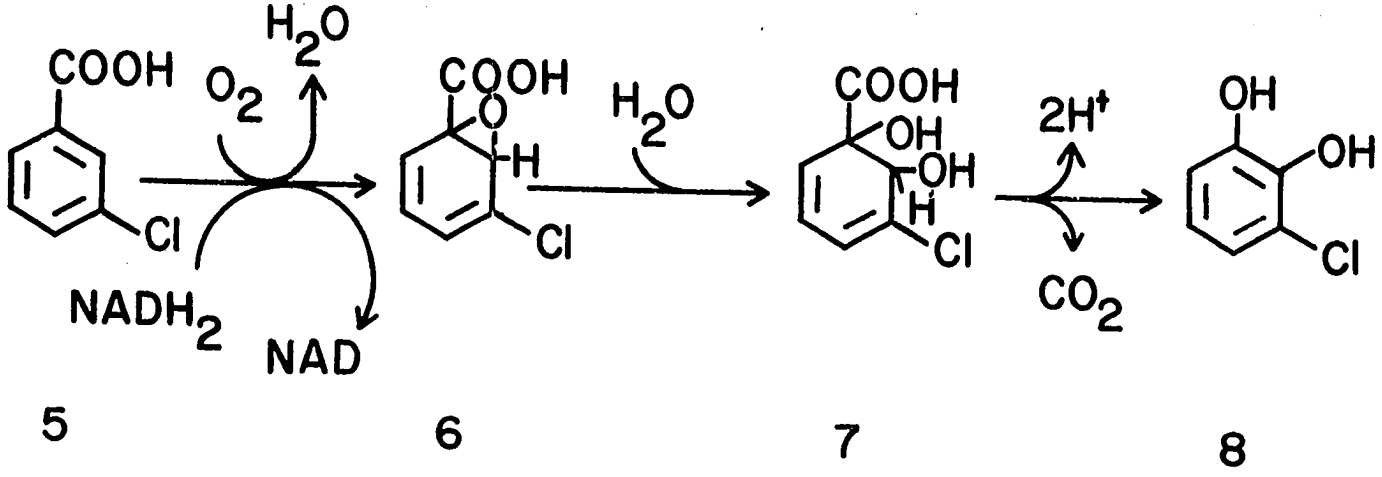
Most studies of dehalogenation have been concerned with the use of pure cultures and relatively little is known of dehalogenation processes as they may occur under natural conditions. Recently, Schreiber, *et al*. (1980) have proposed halogen release mechanisms during fluorobenzoate catabolism by *Pseudomonas* B13.

Catechol properties and conclusion

The production of catechol or catechol derivatives from benzoate or benzoate derivatives during cometabolic reactions more than likely results from the action of a benzoate oxidase system that demonstrates relatively low substrate specificity. As previously mentioned, Ichihara, *et al*. (1962) and Hughes (1965) reported the conversion of benzoate or benzoate derivatives to corresponding catechols. A representation of the conversion of benzoate to catechol and *m*-chlorobenzoate to *m*-chlorocatechol is presented in Figure 6 where it may be noted that a low-substrate-specific benzoate oxidase system active during

Figure 6. The hypothetical mechanism of benzoate (1) and *m*-chlorobenzoate (5) oxidation by a low substrate-specific benzoate oxidase. Initially, one oxygen atom oxidizes either benzoate (1) or *m*-chlorobenzoate (5). The concomitant release of one molecule of water from each oxidation is caused by the reduction of the other oxygen atom. An epoxide type of intermediate (2,6) is generated, which may be hydrated to form a dihydrodiol type of intermediate (3,7). Eventually, two hydrogen protons and one molecule of carbon dioxide are released during each oxidation and the corresponding catechol (4) or chlorocatechol (8) is generated. (Ichihara, *et al.*, 1962)


COOH
O_2
H_2O
$NADH_2$
NAD
COOH
O
H
H_2O
COOH
OH
OH
H
$2H^+$
CO_2
OH
OH
1
2
3
4


COOH
O_2
H_2O
Cl
$NADH_2$
NAD
COOH
O
H
Cl
H_2O
COOH
OH
OH
H
Cl
$2H^+$
CO_2
OH
OH
Cl
5
6
7
8

the conversion of benzoate to catechol may also participate in the production of *m*-chlorocatechol from *m*-chlorobenzoate.

Besides the fact that catechol or catechol derivatives have been observed as accumulated products during several cometabolic processes, one investigator has presented evidence that catechol was responsible for autotoxicity in agar media. The accumulation of catechol by a *Pseudomonas* sp. was reported by Reyrolle (1971), who also presented evidence which suggested that catechol was generally bacteriocidal. Another set of investigators presented evidence that catechol may accumulate in media because of spontaneous transformation to *o*-benzoquinone. This product has been shown to inhibit catechol 1,2-oxygenase activity (Bilton and Cain, 1968).

Scrutiny of the literature has generated information indicating that catechol may be involved in color reactions. For example, Porteus and Williams (1949) isolated catechol from the urine of rabbits that were fed benzene. The color of catechol isolated from these sources was examined under several conditions. In the presence of $FeCl_3$, the color of catechol was intense violet, purple and red-purple at various pH levels. These authors also reported color reactions of catechol in the presence of 2,6-dichloroquinone chloroimide and in the presence of sulfuric acid.

Gibson, *et al.* (1968) also reported of color reactions in supernatants of growth media that contained catechol after *Pseudomonas putida* had grown at the expense of toluene and had cometabolized *m*-chlorobenzoate. These workers assumed that color was caused by the

autooxidation of catechols.

The oxidation of various *m*-substituted benzoates by benzoate oxidase isolated from *Pseudomonas aeruginosa* B-23 grown on benzoate also resulted in the production of a color in reaction mixtures (Ichihara, *et al.*, 1962). A violet color was observed in reaction mixtures containing oxidized products of either *m*-fluorobenzoate, *m*-methylbenzoate or *m*-chlorobenzoate and this color was attributed to the accumulation of catechol derivatives.

During a more recent study, Schreiber, *et al.* (1980) noted a faint violet coloration in a broth medium when chlorobenzoate-grown cells of *Pseudomonas* sp. B13 cometabolized *m*-fluorobenzoate. These authors believed that the violet coloration was due to the formation and accumulation of fluorocatechol.

In the first paper published to implicate catechol as an intermediate in microbial degradation of benzoate, the events that accompany phenol and benzoate metabolism were presented (Evans, 1947). It was noted that intense catechol production was observed when soil bacteria degraded phenol. The presence of *o*-benzoquinone in media containing phenol was also observed during growth studies of soil bacteria, and the implication that *o*-benzoquinone may be involved in color production was made when catechol accumulated in a medium studied by Marr and Stone (1961). In these investigations, soil organisms oxidized benzene and an occasional broth medium turned color. The color was attributed to the presence of catechol and *o*-benzoquinone.

The conversion of catechol to *o*-benzoquinone has been reported by

Dawson and Tarpley (1963) and a color reaction was presented by these authors. Additional evidence for color reactions involving halogenated *o*-benzoquinones in microbial systems has been presented by Crawford, *et al.* (1973) and DiGeronimo, *et al.* (1979).

Although the literature has suggested that catechol or catechol derivatives had been implicated in color reactions, no intensive study had ever been undertaken which verified that these compounds could be incriminated as chromogenic agents which accumulated as the result of a cometabolic process. Therefore, this study advanced in keeping with a philosophy established by Poincare, whereby "It is by logic that we prove, but by intuition that we discover."

EXPERIMENTAL PROCEDURES

Isolation of benzoate-utilizing microorganisms

The broth medium initially used to isolate microorganisms capable of utilizing sodium benzoate (General Chemical Co., New York, N. Y.) contained 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g KH_2PO_4, 1.6 g K_2HPO_4, 0.025 g $FeCl_3 \cdot 6H_2O$, 0.5 g NH_4NO_3 and 0.5 g substrate per liter of distilled and deionized water (Horvath and Alexander, 1970b). To avoid precipitation, salts were added to water in the order described, each being completely dissolved before the addition of the next. Two-hundred-ml portions of this basal salts medium plus sodium benzoate were dispensed in 500-ml Erlenmeyer flasks and each flask was stoppered with a cotton plug. The medium was sterilized by autoclaving at 121°C for 15 min. The pH of the sterile medium was 7.0 and rarely required adjustment.

One flask of sodium benzoate basal salts medium was inoculated with 4.0 g of soil that was freshly-collected from the Iowa State University campus. This mixture was shaken at 150 rpm on a rotary shaker (Model VS, New Brunswick Scientific Co., New Brunswick, N. J.) for two days at room temperature. Two ml were withdrawn from this medium following the initial incubation period and were transferred to a 500-ml Erlenmeyer flask containing 200 ml sterile benzoate basal salts medium. After an additional two-day incubation period under the conditions described, one ml of the medium was diluted in 9 ml of sterile

distilled and deionized water contained in a 16 X 125 mm culture tube. One-tenth ml portions of diluted sample were used to inoculate petri dishes containing benzoate basal salts medium supplemented with 1.5 % agar (Difco). Plates were inoculated by the spread-plate technique and were incubated at 30°C for two days. Pure cultures were obtained by picking 32 different colonies from these plates and streaking them on benzoate basal salts medium containing agar. After two days of incubation at 30°C, colonies were picked and placed into individual 16 X 125 mm culture tubes containing 9.0 ml of sterile benzoate basal salts medium. These tubes were shaken at 150 rpm at room temperature; after two days, 0.1 ml of medium was withdrawn from each tube and transferred to tubes containing 9.0 ml of sterile benzoate basal salts medium. These tubes were incubated for two days under the same conditions as the initially-inoculated tubes. After this incubation period, all 32 isolates continued to grow at the expense of sodium benzoate.

Stock cultures of each isolate were prepared after organisms were streaked on benzoate basal salts plus agar medium. The strains were maintained on slopes of benzoate basal salts plus agar medium contained in 16 X 125 mm screw-capped tubes. Seven ml of the agar-based medium were placed into each tube to prepare slopes and after the tubes were inoculated and incubated at 30°C for two days, luxuriant growth resulted. Stock cultures of the isolates were prepared in this manner and were stored at 4°C. Organisms were transferred once monthly.

The use of chlorinated benzoic acids as sole carbon and energy sources by benzoate-utilizing microorganisms

Five broth media and five solid media were employed to establish whether or not benzoate-utilizing microorganisms could use chlorinated benzoic acids as sole carbon and energy sources for growth. Each of the broth media consisted of the basal salts medium previously described and contained either 2,3,6-trichlorobenzoic acid (technical grade, AMCHEM Products, Inc., Agricultural Chemicals Division, Ambler, PA., donated by Dr. David W. Staniforth, Dept. Plant Pathology, Iowa State University), 2,4-dichlorobenzoic acid (reagent grade, Eastman Kodak Co., Rochester, N. Y.), 2-(*o*-)chlorobenzoic acid, 3-(*m*-)chlorobenzoic acid or 4-(*p*-)chlorobenzoic acid as sole sources of carbon and energy. All chlorinated benzoic acids were used at concentrations of 0.5 g per liter of medium, and all monochlorinated analogs of benzoic acids were of reagent grade and were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Additional supplies of 3-chlorobenzoate were also obtained from Sigma Chemical Co., St. Louis, MO.

Nine ml of each broth medium containing a chlorinated benzoic acid were dispensed into 16 X 125 mm culture tubes and were sterilized by autoclaving. The pH of all five sterile media was 7.0. Each tube of medium was inoculated with a loopful of a fresh culture of cells growing on sodium benzoate basal salts medium plus agar. The tubes were then shaken at 150 rpm at room temperature. Growth was monitored daily for one week by measuring optical density (O.D.) at 510 nm

with a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, N. Y.). After one week, one-tenth ml of each tube was transferred to tubes containing respective chlorinated benzoic acid basal salts media. The tubes were monitored for growth and catechol production for a one-week period.

To test for the ability of benzoate-utilizing organisms to use chlorinated benzoates as sole sources of carbon and energy, five solid, agar-based media were also employed. Each medium consisted of basal salts medium prepared as previously described and was supplemented with 1.5 % agar (Difco). Chlorinated benzoic acids were added at a concentration of 0.5 g per liter of medium; 2,3,6-trichloro-, 2,3-dichloro-, *o*-chloro, *m*-chloro and *p*-chlorobenzoic acids were used. Solid media were sterilized in an autoclave and dispensed into petri dishes. The plates were dried overnight at 37°C and a loopful of each fresh culture of cells growing on benzoate basal salts plus agar was used to inoculate plates containing chlorinated benzoate media. These plates were incubated at 30°C and were examined over a two-week period.

Selection and identification of *P. fluorescens*

Although none of the organisms was capable of growing at the expense of any of the chlorinated benzoic acids investigated, most of the organisms produced catechol in media containing *m*-chloro-benzoate. One of these organisms produced significantly high amounts of catechol in the medium containing only *m*-chlorobenzoate and this organism was chosen for further study. No catechol was produced by

any organisms placed in media containing chlorinated benzoates other than *m*-chlorobenzoate.

The organism selected for investigation of cometabolic capabilities was a Gram-negative, rod-shaped bacterium that was strictly aerobic. The bacterium was highly motile, produced catalase, was oxidase positive and did not accumulate poly-*B*-hydroxybutyrate. Because of other characteristics examined and the ability of this organism to fluoresce, the bacterium was identified as a strain of *Pseudomonas fluorescens* according to criteria established in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (Buchanan and Gibbons, 1974).

Verification of aromatic ring-cleavage mechanisms

In order to verify the mechanism of ring cleavage utilized by *P. fluorescens*, a test described by Holding and Collee (1971) was performed. A loopful of cells from an actively-growing culture of the bacterium was transferred to 18 X 150 mm culture tubes containing 3.5 mM sodium benzoate plus basal salts broth. The organism was incubated with shaking at 30°C, and after good growth was obtained (at about 6 h), cells were removed by centrifugation and were resuspended in 2.0 ml of 0.02 M tris buffer (pH 8.0) contained in 16 X 125 mm culture tubes. Five-tenths ml of toluene and 20.0 *u*moles of protocatechuic acid were then added to the cell suspension and the tubes were shaken at room temperature. The development of a bright yellow color within a few minutes indicated that *ortho*-cleavage of the substrate had taken place. If the result was negative, tubes

were shaken for an hour at 30°C before the Rothera reaction was performed to test for *ortho*-cleavage. After shaking, 1.0 g of $(NH_4)_2SO_4$ crystals was added to each tube, followed by one drop of a 1.0% (w/v) sodium nitroprusside solution. Tubes were gently mixed and about 0.5 ml of 0.9 sp gr NH_4OH was added to the solution. After mixing, development of a deep violet color due to the presence of *b*-ketoadipic acid indicated ortho-cleavage of the substrate.

Catechol assay

A modified version of the method originally established by Arnow (1937) was employed to measure the concentration of catechol. Three reagents were required for this assay and they were prepared in 200-ml portions in distilled and deionized water to which was added either (1) 8.32 ml of concentrated HCl (which resulted in a 0.5 N HCl solution), (2) 20.0 g of $NaNO_2$ and 20.0 g of $NaMoO_4 \cdot 7H_2O$ or (3) 8.0 g of NaOH (which resulted in a 1.0 N solution). The reagents were prepared in three 250-ml Erlenmeyer flasks; following preparation, each flask was covered with Parafilm (American Can Co., Greenwich, CT.) and stored at room temperature. Catechol standard curves were produced by analyzing solutions containing between 0 and 36.0 *ug* of catechol (pyrocatechol, Eastman Kodak Co., Rochester, N. Y.) per ml of distilled and deionized water. Standard curves were prepared each day catechol assays were performed by plotting absorbance at 510 nm on the ordinate and concentration of catechol in *ug*/ml on the abscissa of linear graph paper. In instances where catechol was assayed in growth media or resting cell media, samples

were removed by centrifugation before catechol levels were determined.

Reaction tubes were prepared by adding 1.0 ml of sample or sample diluted in distilled and deionized water to each of several 13 X 100 mm culture tubes. One ml of 0.5 N HCl was then added to each tube and the contents were vortex-mixed thoroughly. One ml of $NaNO_2$-$NaMoO_4 \cdot 7H_2O$ solution was then added to the acidified sample at which time a bright yellow color formed if catechol was present in the sample. The contents were mixed and 1.0 ml of 1.0 N NaOH was added to each culture tube. At this point, a bright pink to red color appeared, depending on the concentration of catechol present. This mixture was thoroughly mixed and allowed to stand at room temperature for approximately one minute to allow the reaction to proceed fully. After this incubation period, the total 4.0 ml contained in each tube was transferred to 13 X 100 mm cuvettes and the optical density of the reaction mixtures was determined at 510 nm in a Spectronic 20 spectrophotometer.

Assay of chloride ions

Chloride ion determinations were performed by using a chloride ion-sensing electrode (Model IS-146, Lazar Research Laboratories, Los Angeles, CA.) attached to an expanded scale pH/millivolt meter (Model 76, Beckman Instruments, Inc., Fullerton, CA.). When chloride ions were assayed in broth culture media, it was not necessary to centrifuge the growth media and 25 ml were usually sufficient for each assay. Samples were placed into 50.0-ml beakers and were stirred during the time measurements were obtained. To allow the

expanded scale pH/millivolt meter to equilibrate, chloride ions were measured continuously for 5 min.

Standard curves were prepared by using NaCl that had been dried for several days at 70°C under vacuum. Chloride ion standards were prepared by mixing dried NaCl with distilled and deionized water, and concentrations of standards ranged from 5.0×10^{-4} M to 1.0×10^{-2} M chloride ions. Twenty-five ml of each standard were placed in 50.0-ml beakers and stirred with a magnetic stirring bar. The chloride ion-sensing electrode and a standard reference electrode were introduced into the NaCl standard and readings were obtained in (+) millivolts. Standard curves were obtained by plotting (+) millivolts along the abscissa and chloride ion concentration (mmoles/l) along the ordinate of semi-logarithmic graph paper.

Initial sodium benzoate growth studies

Initial studies were performed to examine the growth characteristics of *P. fluorescens* in broth media when sodium benzoate was supplied as a sole source of carbon and energy. Each broth medium contained 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g KH_2PO_4, 1.6 g K_2HPO_4, 0.045 g $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ 0.5 g NH_4NO_3 and 3.5 mmoles or 7.0 mmoles sodium benzoate per liter of distilled and deionized water. Media were sterilized after 500.0 ml were placed into 1.0-liter Erlenmeyer flasks.

Inocula for all growth studies were prepared by inoculating 18 X 150 mm culture tubes containing 9.0 ml of 3.5 mM sodium benzoate plus basal salts with a loopful of a fresh culture of *P. fluorescens* growing on sodium benzoate basal salts plus agar slopes. Inocula

were incubated at 30°C overnight with shaking until an O.D. of 0.8 was observed at 510 nm.

Flasks containing 500.0 ml of growth media were inoculated with 5.0 ml of inoculum and the growth of *P. fluorescens* was assayed by measuring O.D. at 510 nm and by determining viable cell numbers.

Catechol was assayed via the method of Arnow (1937). Substrate disappearance was verified via ultraviolet spectroscopy when one ml of culture medium was diluted 1:10 in distilled and deionized water and a decrease in absorbance was measured with a Beckman spectrophotometer, Model DB (Alexander and Lustigman, 1966).

The optical density of growth media was monitored with a Spectronic 20 spectrophotometer. Four ml of medium were withdrawn from growth vessels and placed into 13 X 100 mm cuvettes. When necessary, appropriate dilutions of growth media were made in distilled and deionized water.

Viable cell numbers were determined by plating samples or diluted samples of growth media on Trypticase Soy Agar (TSA, Baltimore Biological Laboratory, Division Becton, Dickinson and Company, Cockeysville, MD.) contained in petri plates. Dilutions were made in sterile, 9.0-ml dilution blanks consisting of distilled and deionized water plus 0.01 % peptone and were contained in 16 X 125 mm culture tubes. One-tenth ml of sample or diluted sample was placed on the surface of the TSA and the spread-plate technique was used to inoculate all plates. Incubation was allowed for at least 24 h at 30°C and colonies were counted by using a darkfield Quebec colony counter (Model 3325,

American Optical, Scientific Instrument Division, Buffalo, N. Y.).

Initial cometabolism studies

As stated previously, it is impossible to enrich for organisms that are capable of cometabolizing non-utilizable substrates, but to isolate organisms on easily-catabolized analogs of recalcitrant molecules is readily accomplished. Once isolated, the cometabolic abilities of an organism can be examined by adding recalcitrant compounds to a medium containing the degradable substrate. During the course of growth, it is necessary to monitor media for the presence of suspected cometabolites.

Initially, studies performed to determine the capacity of *P. fluorescens* to cometabolize chlorinated benzoates included the examination of media containing 2,3,6-tri-, 2,4-di-, *o*-, *m*- or *p*-chlorobenzoate. Each of these potentially cometabolizable substrates was added at a concentration of 3.5 mmoles per liter of basal salts medium containing 3.5 mmoles sodium benzoate. Media were prepared in 500-ml volumes, dispensed into 1-liter Erlenmeyer flasks and sterilized by autoclaving. Media were inoculated with a 1.0 % inoculum that had been prepared as described previously. Catechols were assayed during these growth studies and O.D. was measured to determine growth. It was evident from these growth studies that *P. fluorescens* could cometabolize only *m*-chlorobenzoate, since catechols were not detected and did not accumulate in media containing chlorinated benzoates other than *m*-chlorobenzoate. A striking chromogenic reaction

took place when culture media containing benzoate and *m*-chlorobenzoate were incubated over a 12-h period. After this period of incubation, an intense purple color appeared in the media and it was also noted that catechols were detectable by the Arnow test and accumulated in the media. It was because of marked color production and associated catechol accumulation that this system was studied in detail.

Growth studies of the cometabolism of *m*-chlorobenzoate

To assess the cometabolic capabilities of *P. fluorescens*, growth studies were performed by using basal salts broth medium containing: (1) *m*-chlorobenzoate, (2) *m*-chlorobenzoate plus sodium benzoate or (3) sodium benzoate. All carbon sources were supplied at levels of 3.5 mmoles per liter of basal salts, prepared in 500-ml volumes and placed in 1.0-liter Erlenmeyer flasks. These media were sterilized in an autoclave and were inoculated with 5.0 ml of an overnight inoculum of *P. fluorescens*. Culture media were incubated at 30°C with shaking at 150 rpm and samples were collected at regular intervals.

Growth was assayed by monitoring O.D. at 510 nm and by performing viable cell counts after plating samples on TSA. Catechol levels and chloride ions were also assayed. The increase in purple color was measured by monitoring the O.D. at 510 nm of media that had been clarified by centrifugation.

Growth studies were also performed by using broth media consisting of glucose and glucose plus *m*-chlorobenzoate. In these studies, growth of *P. fluorescens* was assayed by measuring viable cell numbers

and catechol levels were determined. Media were prepared in 1-liter Erlenmeyer flasks containing 500 ml of basal salts plus 3.5 mM *m*-chlorobenzoate. Media were sterilized in an autoclave. Glucose was sterilized independently as a 10.0 % solution of the carbon source dissolved in distilled and deionized water. The sterile glucose solution was added to the previously-sterilized basal salts solution to result in a final concentration of 3.5 mmoles glucose per liter of medium.

Cometabolism of *m*-chlorobenzoate by *P. fluorescens* grown on alternative aromatic carbon sources

In this study, media consisting of carbon sources other than sodium benzoate, but degraded via the *B*-ketoadipic acid pathway were examined. Basal salts media containing 3.5 mM shikimic acid, quinic acid or vanillic acid were prepared in 500.0 ml quantities. These media were also supplemented with 3.5 mM *m*-chlorobenzoate and were placed in 1.0-liter Erlenmeyer flasks and sterilized in an autoclave. Each medium was inoculated with 5.0 ml of *P. fluorescens* grown overnight in benzoate basal salts. Chromogenesis was monitored qualitatively and only catechols were assayed during this study.

Growth studies of *P. fluorescens* during the cometabolism of *m*-chlorobenzoate at various levels of sodium benzoate

These studies employed basal salts media in which the initial concentration of *m*-chlorobenzoate was always 3.5 mmoles per liter of medium, but the concentrations of sodium benzoate were either 3.5, 2.625, 1.75 or 0.875 mmoles per liter of medium. These studies

were performed to determine whether or not the amount of sodium benzoate available for growth would affect the rate and extent of production of accumulated catechols and corresponding purple color. The effects of various substrate levels on the rate of disappearance of accumulated catechol and the extent of dechlorination were also investigated.

Eight media were employed during this study, including the four media described in the previous paragraph plus four media consisting of sodium benzoate as the only substrate at levels of 3.5, 2.625, 1.75 and 0.875 mmoles per liter of basal salts. Media were prepared in 600-ml volumes and were placed in 1-liter Erlenmeyer flasks. The media were sterilized in an autoclave and were inoculated with a 1.0 % inoculum. Cultures were incubated with shaking at 30°C. Samples were collected at regular intervals and growth was determined by measuring O.D. at 510 nm. Catechol levels were assayed via the method of Arnow and chloride ions were detected by using methods previously described.

Growth of *P. fluorescens* during the cometabolism of *m*-chlorobenzoate in the absence and presence of iron

Because a relationship between chromogenesis of catechol and ferric ions had been presented in the literature and had been recognized during the studies described in this dissertation, growth studies were performed to determine the effect of iron on the production of the chromogenic cometabolite. These studies were conducted by using the basal salts medium that was used in all previous cometabolism

studies, but in order to determine the effect of the absence of iron in the media, $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ was omitted from the composition.

Two media were used in this study and included 3.5 mmoles sodium benzoate and 3.5 mmoles *m*-chlorobenzoate per liter of basal salts and 3.5 mmoles sodium benzoate plus 3.5 mmoles *m*-chlorobenzoate per liter of basal salts devoid of $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$. Media were prepared in 600-ml volumes, placed in 1-liter Erlenmeyer flasks and were sterilized by autoclaving. Six ml of inoculum were added to each flask and both media were incubated with shaking at 30°C. Growth was determined by measuring O.D. at 510 nm, and chloride ion release as well as catechol production were determined. Purple color production was determined by methods previously described.

Growth of *P. fluorescens* in media containing sodium benzoate and supplemented with catechol or 3-chlorocatechol

Several growth studies were performed to determine whether or not catechol or 3-chlorocatechol were toxic to cells of *P. fluorescens*. In one study, basal salts media contained sodium benzoate and were supplemented with three levels of catechol. Since media containing catechol turned black during sterilization by autoclaving, catechol was sterilized by filtration (Gelman acrodisc disposable filter assembly, 0.45 μm pore size, Gelman Sciences, Inc., Ann Arbor, MI.) and added to previously-sterilized media.

Four media were employed in one study that investigated the effect of various concentrations of catechol on *P. fluorescens* growing on

sodium benzoate. These media contained 3.5 mmoles sodium benzoate per liter of basal salts and catechol was added at concentrations of 0, 0.25, 0.5 and 2.0 mmoles per liter of medium. Media were prepared in 500-ml quantities and were placed in 1-liter Erlenmeyer flasks. Each medium was inoculated with 5.0 ml of *P. fluorescens* previously grown in sodium benzoate plus basal salts. In this study, growth was measured by observing O.D. at 510 nm and by determining viable cell numbers on TSA. Catechol was assayed via the method of Arnow.

In a study designed to determine whether or not catechol or 3-chlorocatechol could serve as growth substrates for *P. fluorescens*, four media were employed; they contained 3.5 mmoles or 1.75 mmoles of 3-chlorocatechol or 3.5 mmoles or 1.75 mmoles of catechol per liter of basal salts medium. The media were prepared in volumes of 62.5 ml and were dispensed in 250-ml Erlenmeyer flasks and sterilized by autoclaving. The catechols were added to sterile basal salts after being sterilized by filtration. Each flask of medium was inoculated with 0.62 ml of *P. fluorescens* grown overnight in benzoate and basal salts broth medium. Viable cell numbers were determined by plating 0.1 ml of sample or diluted sample on plates of TSA. During the first two hours of the growth study, samples were collected every hour, and samples were collected at less frequent intervals during a 48-h period following inoculation. Catechol levels were also determined during the course of this study.

The sequential addition of 3-chlorocatechol to a basal salts medium containing 3.5 mM sodium benzoate was also investigated to

determine whether or not an actively-growing culture of *P. fluorescens* would be able to completely degrade 3-chlorocatechol. The basal salts medium containing 3.5 mmoles sodium benzoate per liter was prepared in 500-ml quantities and were placed in 1-liter Erlenmeyer flasks. sterilized. Ten ml of a solution containing 3-chlorocatechol contained in distilled and deionized water was prepared and sterilized by filtration (Gelman acrodisc disposable filter assembly, 0.45 um pore size, Gelman Sciences, Inc., Ann Arbor, MI.). The 3-chlorocatechol was added to the basal salts medium containing sodium benzoate at 2, 4, 6, 8 and 10 h during the growth study to result in final concentrations of 0.7, 1.4, 2.1, 2.8 and 3.5 mmoles of 3-chlorocatechol per liter of medium at each respective time of addition. The medium was inoculated with 5.0 ml of *P. fluorescens* grown on benzoate basal salts medium and the cultures were incubated with shaking at 30°C. Growth was monitored by determining viable cell numbers after samples were plated on plates of TSA. Levels of catechol and chloride ions were also determined during this study.

Chromogenesis disappearance study

To more closely examine the details of purple utilization as previously evidenced in growth studies of *P. fluorescens* grown in a medium containing both sodium benzoate and *m*-chlorobenzoate, a study was designed to qualitatively investigate the disappearance of purple color. Several carbon sources, including sodium benzoate, protocatechuic acid, glucose, catechol and fumarate were used in this study. These

carbon sources were added to basal salts broth medium to result in a concentration of 3.5 mmoles per liter of medium. These media were prepared in 75-ml portions and were placed in 125-ml Erlenmeyer flasks. The media were inoculated with a 1.0 % inoculum of *P. fluorescens* and incubated with shaking at 30°C. After the organism had reached mid-log phase in each culture medium, *m*-chlorocatechol that had previously been allowed to react in a solution of $Fe(NH_4)_2SO_4 \cdot 12H_2O$ to become purple was added to each flask to result in final concentrations of either 0.21 or 0.035 mmoles of the catechol per liter of medium. Purple color was qualitatively observed over a two-day time period to note persistence or disappearance.

Resting cell studies

Studies were performed in order to determine whether or not *P. fluorescens* could convert *m*-chlorobenzoate to catechols under resting cell conditions. Cells of *P. fluorescens* were obtained by growing the organism in basal salts medium containing 3.5 mmoles of sodium benzoate and 3.5 mmoles of *m*-chlorobenzoate per liter of medium. The growth medium was prepared in two portions of 500 ml each and placed into two 1-liter Erlenmeyer flasks. The medium was sterilized by autoclaving and each flask was eventually inoculated with 5.0 ml of *P. fluorescens* actively growing at the expense of sodium benzoate contained in basal salts medium.

Resting cell media were comprised of basal salts and either 3.5 mM or 1.75 mM *m*-chlorobenzoate. One set of each sterile medium was also supplemented with 0.25 mg/ml NADH (nicotinamide adenine dinucleotide,

reduced, Sigma Chemical Co., St. Louis, MO.) which had been previously sterilized as a solution in distilled and deionized water by filtration. The resting cell media were dispensed in 4.5-ml portions and were contained in sterile 18 X 150 mm culture tubes.

Cells from the sodium benzoate plus *m*-chlorobenzoate growth medium were harvested at 3, 6, 9 and 12 h of growth by centrifugation at 2000 X *g* and were resuspended in sterile basal salts medium. The O.D. of the suspension was adjusted to 0.2, and 0.5 ml of the suspension was added to each resting cell medium. The resting cell media were then examined for catechol production and chloride ion release at 30 min, 1, 2, 8 and 12 h after the addition of resting cells to the reaction mixtures.

Synthesis of 3-chlorocatechol and 4-chlorocatechol

Because accumulated catechol-positive material was observed in cometabolism studies described in this dissertation and because the literature described the accumulation of chlorinated catechols in previous studies of cometabolism of *m*-chlorobenzoate, it was necessary to obtain authentic 3-chloro- and 4-chlorocatechol. Since neither compound is commercially available, they were synthesized in the laboratory.

The method used to synthesize the chlorocatechols was modified from that originally published by Willstätter and Müller (1911). The synthesis was initiated when 125.0 g of catechol (pyrocatechol, crystalline, Sigma Chemical Co., St. Louis, MO.) was chilled to $-5^{\circ}C$

and added to 500 ml of anhydrous diethyl ether (Fisher Scientific Co., Fairlawn, N. J.) that had been chilled to -5°C and placed in a 1-liter beaker. This addition step was performed with stirring in an ice bath mounted on a magnetic stirrer. Ninety-five ml of sulfuryl chloride (SO_2Cl_2, Mallinckrodt, Science Products Div., Mallinckrodt Inc., St. Louis, MO.) were chilled to -5°C and were added dropwise with continuous mixing to the chilled, diethyl ether-catechol mixture. The addition of sulfuryl chloride was performed over a 5-hour period so that the temperature of the reacting mixture did not rise above 5°C.

After complete addition of sulfuryl chloride, mixing was continued for 1 h at 1°C. The solvent was then removed from the mixture by evaporation (flash evaporator, Buchler Instruments, Fort Lee, N. J.) at approximately 10 mm Hg. This process was allowed to occur at 50°C for 12 h until approximately 50 ml of solvent remained in the mixture. A copious amount of crystals (4-chloro-catechol) was produced during this procedure.

The crystals were transferred to a 500-ml separatory funnel and 50 ml of warm (50°C) benzene and a few glass beads were added to the mixture. The separatory funnel and contents were shaken by hand for 15 min after which 150 ml of petroleum ether B (Chemistry Stores, Iowa State University) were added and the solution and crystals were transferred to a 1-liter beaker and stirred for several hours at room temperature.

The mixture was then filtered and crystals were washed several times with a solution consisting of equal volumes of petroleum ether

B and benzene. These crystals were air-dried after being placed into a baking dish. After drying, the crystals were stored under nitrogen in a tightly-closed glass container.

All but 75 ml of solvent remaining from the crystallization was removed from the filtrate by evaporation under the same conditions previously described in the procedures of this synthesis. The remaining solution was distilled under vacuum at 2 mm Hg and fractions were collected at 85-95°C. After collection, liquid fractions of 0.5 ml each were placed in lyophile tubes and the contents were lyophilized (VirTis Research Equipment, Gardiner, N. Y.) for approximately 6 h to remove water from the highly-deliquescent 3-chlorocatechol. The compound was stored in sealed lyophile tubes under vacuum at room temperature in the dark.

Verification of 3-chlorocatechol was provided by proton nuclear magnetic resonance (NMR) spectroscopy (Hitachi-Perkin Elmer model R20B, 60 mhz or E. M. Varian model 360, 60 mhz NMR spectrometers; all chemical shifts were noted in ppm, delta scale from tetramethyl silane), infrared spectroscopy (Beckman model IR 4250) and mass spectrometry (AEI model MS902 mass spectrometer). 4-Chlorocatechol was identified via proton NMR and infrared spectroscopy. Melting point determinations of each compound were also made.

Extraction and identification of accumulated catechols from growth media

The identification of chlorinated catechols presented several analytical problems because the compounds exhibited volatility, were easily oxidized and 3-chlorocatechol was extremely hydroscopic.

These difficultues were overcome, in part, when the chlorinated catechols were precipitated with divalent lead salts according to the methods described by Helling and Bollag (1968). To prepare lead catecholates, resting cell suspensions of *P. fluorescens* were initially used to produce the cometabolite. Two liters of basal salts medium containing 3.5 mM sodium benzoate and 3.5 mM *m*-chlorobenzoate were prepared and 500-ml portions were placed in 1-liter Erlenmeyer flasks. This medium was sterilized in an autoclave and eventually each 500 ml of the medium was inoculated with an overnight culture of *P. fluorescens* grown on benzoate basal salts medium.

The medium containing sodium benzoate and *m*-chlorobenzoate was inoculated and the organism was allowed to grow for 6 h at 30°C with shaking. After this incubation period, catechol was detectable in the medium. The cells were removed by centrifugation at 2000 X g and resuspended in 20 ml of basal salts containing 3.5 mM *m*-chlorobenzoate. The cells were then added to 500 ml of basal salts plus 3.5 mM *m*-chlorobenzoate contained in a 1-liter Erlenmeyer flask. The resulting medium was shaken at 30°C for 8 h; a strong catechol reaction was obtained by using the Arnow test. The cells were then removed from the medium by centrifugation at 2000 X g.

To remove residual proteins from the clarified resting cell medium, 20.0 ml of a 10 % (w/v) tungstic acid solution contained in 0.083 N H_2SO_4 were added to the sample. Tungstic acid was prepared by mixing 50.0 g of sodium tungstate (General Chemical Division,

Allied Chemical and Dye Corp., New York, N. Y.) with 50 ml of concentrated HCl. The tungstic acid was removed from the HCl via centrifugation and the yellow compound was washed several times with distilled and deionized water to remove NaCl and residual HCl. The tungstic acid was dried at room temperature before use. The tungstic acid-treated medium was centrifuged to remove proteins and insoluble acid. The supernatant was filtered through filter paper and extracted 3 X with of diethyl ether. The ether phase was concentrated by using a stream of nitrogen gas and then mixed with an equal volume of 10 % (w/v) lead acetate ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$) in N_2-purged distilled and deionized water. The precipitate was washed two times with water, dried under vacuum at 70°C for 1 h and stored in a desiccator under vacuum.

Infrared spectra were obtained using a Beckman Model IR 4250 infrared spectrophotometer. Samples were analyzed by preparation of pellets with KBr. In addition, samples were collected before catechols were precipitated with lead acetate and were analyzed by both ultraviolet and proton NMR spectroscopy.

Examination of catechols by color reactions

Basal salts medium containing 0.2 g $MgSO_4 \cdot 7H_2O$, 0.4 g KH_2PO_4, 1.6 g K_2HPO_4, 0.045 g $Fe(NH_4)SO_4 \cdot 12H_2O$ and 0.5 g NH_4NO_3 per liter of distilled and deionized water were prepared and 25.0 ml were dispensed into each of several 50-ml Erlenmeyer flasks. The basal salts medium was sterilized in an autoclave and either catechol extracted from resting cells, catechol standard, 3-chlorocatechol or 4-chlorocatechol was added to each flask to result in a final concentration

of 25.0 ug/ml. The flasks were shaken at room temperature for 12 h until color reactions had occurred. The pH values of these media were adjusted to 1.5, 6.0, 7.0, 8.0 and 9.0; colors were noted at each pH level. Each medium was also examined by ultraviolet spectroscopy. The color reactions of catechols were also noted after treating each sample with concentrated sulfuric acid. Samples were also allowed to react for one week and these media were examined for the possible production of insoluble products.

To detect chromogenesis of catechols in basal salts media, another study was performed. Each constituent salt of the basal salts medium was used independently or in every possible combination to determine if any specific salts were responsible for the chromogenic properties of catechols. Salts and respective combinations were used in the concentrations typically used in all growth studies and were dissolved in distilled and deionized water. Ten ml were placed into each of 16 X 125 mm culture tubes. The catechols were added at a concentration of 2.0 mmoles per liter of medium. The mixtures were allowed to equilibrate at room temperature and colors were noted during a 36-h time period.

Assay of benzoquinones

The production of benzoquinones was investigated by methods modified after those described by Marr and Stone (1961). This was accomplished by adding 1.0 ml of aniline to media after 24 h of growth. Ether extraction was used to crystallize red needles which are indicative of benzoquinones.

RESULTS

The use of chlorinated benzoic acids as sole carbon and energy sources by benzoate-utilizing microorganisms

After isolating 32 microorganisms from soil that were capable of utilizing sodium benzoate as a sole source of carbon and energy for cell growth and maintenance, the capacity of each microorganism to catabolize chlorinated benzoic acids was examined. None of the microorganisms were capable of growing at the expense of either 2,4-di-, 2,3,6-tri-, *o*-, *m*- or *p*-chlorobenzoic acids, as evidenced by no increase in O.D. at 510 nm in broth media over a one-week period. In addition, no growth of any microorganisms occurred after the organisms were incubated on solid agar media containing any chlorinated benzoic acid. A catechol-positive material was detected via the Arnow method only in culture tubes containing basal salts broth medium supplemented with *m*-chlorobenzoate. The levels of catechol-positive material observed in this medium are presented in Table 2. A black precipitate was also observed after 72 h in culture tubes containing a basal salts broth medium supplemented with *m*-chlorobenzoate.

Microorganism # 6 (Table 2) produced 0.12 mmoles of catechol-positive material per liter of medium, and it was chosen for use in further investigations. For ease of presentation of some experimental procedures, properties of this organisms were summarized

Table 2. The accumulation of catechol-positive material (as detected via the Arnow method) in culture tubes containing basal salts broth medium supplemented with *m*-chlorobenzoate. Each tube of medium was inoculated with one of 32 organisms isolated from soil and capable of utilizing sodium benzoate as a sole source of carbon and energy.

Organism	Catechol (mmoles/l)	Organism	Catechol (mmoles/l)	Organism	Catechol (mmoles/l)
1	0.06	12	0.00	23	0.04
2	0.02	13	0.03	24	0.02
3	0.00*	14	0.03	25	0.05
4	0.08	15	0.00	26	0.01
5	0.06	16	0.03	27	0.08
6	0.12	17	0.03	28	0.01
7	0.04	18	0.03	29	0.03
8	0.03	19	0.03	30	0.03
9	0.00	20	0.09	31	0.02
10	0.00	21	0.03	32	0.01
11	0.10	22	0.07		

*The lower limit of detection of catechol using the Arnow method was 0.01 m moles per liter.

in Experimental Procedures. As stated previously, this organism was identified as *Pseudomonas fluorescens*.

Verification of aromatic ring-cleavage mechanisms

Enrichment culture techniques had previously confirmed that *P. fluorescens* was capable of utilizing sodium benzoate as a sole source of carbon and energy. To determine the exact mechanism of aromatic ring cleavage exhibited by this bacterium, the Rothera test (which detects the presence of a keto group) was performed. This test indicated that *P. fluorescens* possessed enzymes of the *ortho*-cleavage branch of the *B*-ketoadipic acid pathway (Figures 1 and 2) because a deep violet color developed in reaction tubes due to the presence of *B*-ketoadipic acid.

Initial sodium benzoate growth studies

The results of studies performed to examine the growth characteristics of *P. fluorescens* in basal salts broth medium supplemented with either 3.5 or 7.0 mmoles of sodium benzoate per liter are presented in Figure 7. In addition, the levels of catechol detected during growth of *P. fluorescens* in media containing these two levels of the carbon source are presented in Figure 8.

Initial cometabolism studies

During initial cometabolism studies, *P. fluorescens* was grown in media containing 3.5 mmoles sodium benzoate per liter of basal salts supplemented with 3.5 mmoles per liter of

Figure 7. The growth of *P. fluorescens* in basal salts broth medium supplemented with either 3.5 mmoles (closed circles) or 7.0 mmoles (open circles) of sodium benzoate per liter of medium. Media were inoculated with a 1.0 % inoculum of the bacterium and were incubated at 30°C with shaking. Growth was determined by measuring viable cell numbers (solid lines) and O. D. (broken lines) at 510 nm.

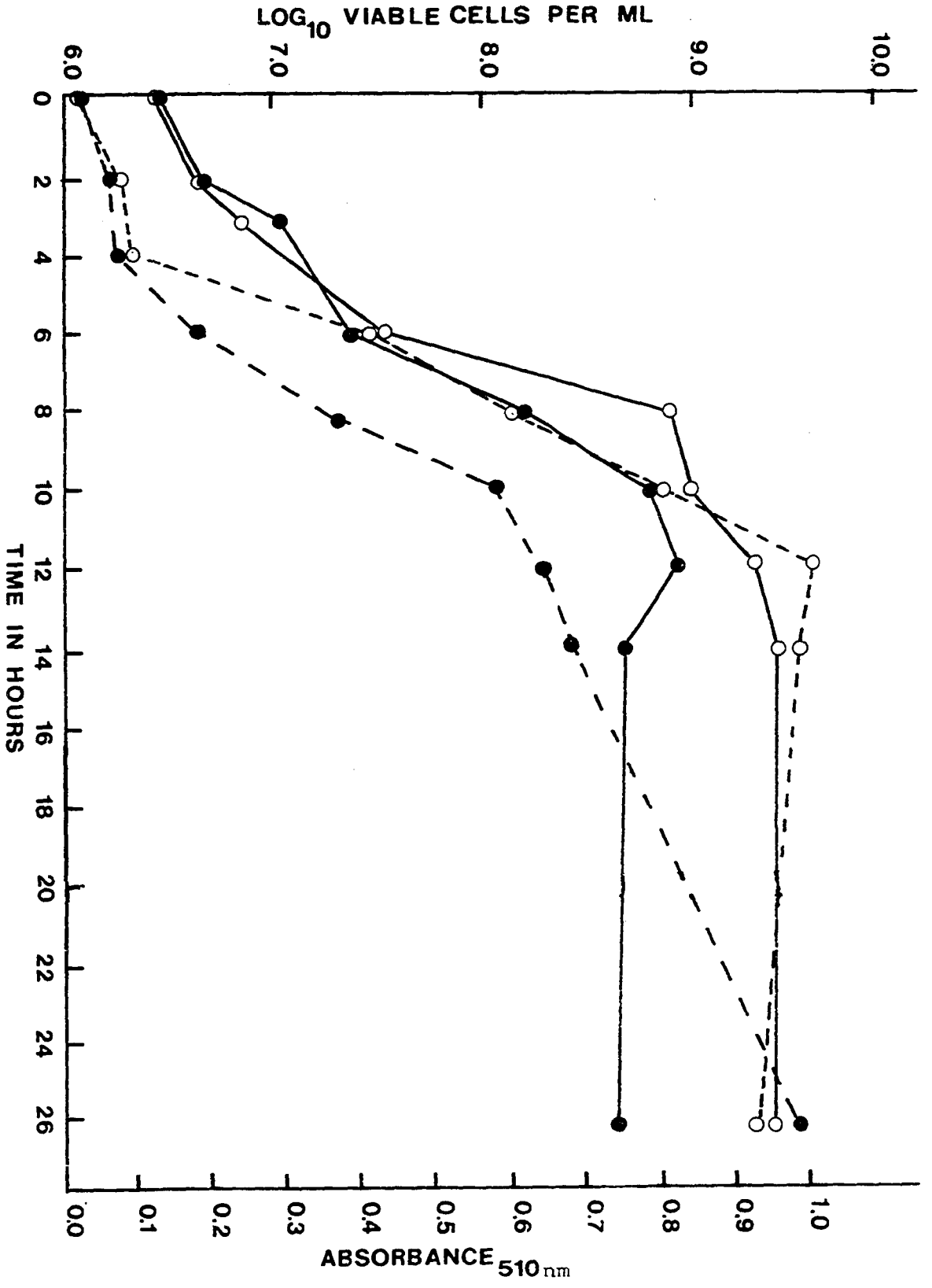
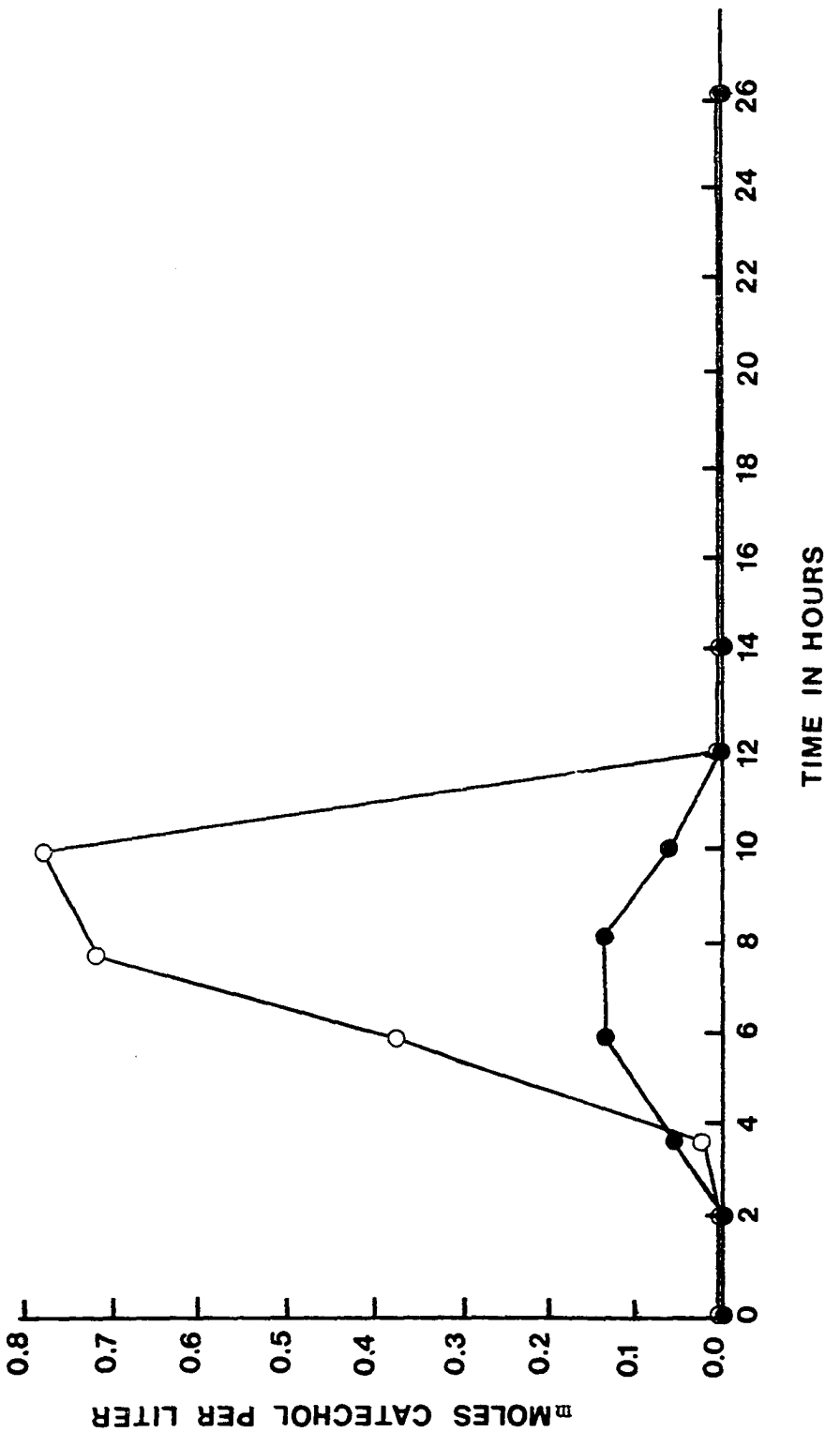

LOG10 VIABLE CELLS PER ML
6.0
7.0
8.0
9.0
10.0
TIME IN HOURS
0
2
4
6
8
10
12
14
16
18
20
22
24
26
ABSORBANCE 510 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

Figure 8. Catechol levels detected in basal salts broth supplemented with either 3.5 (closed circles) or 7.0 (open circles) mmoles of sodium benzoate per liter of medium. Media were inoculated with a 1.0 % inoculum of *P. fluorescens* and were incubated at 30°C with shaking. Catechol was detected via the Arnow method.

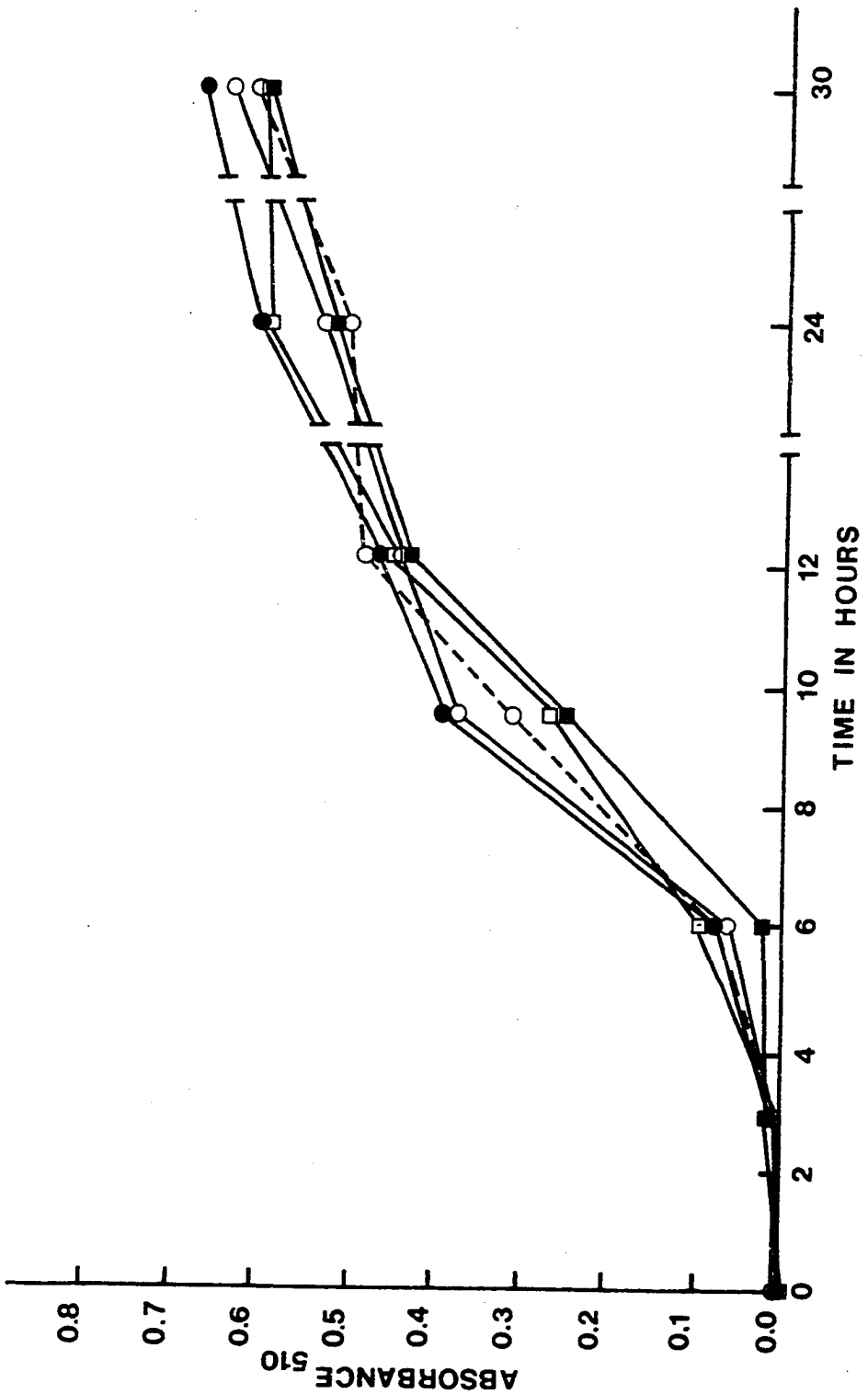

μMOLES CATECHOL PER LITER
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME IN HOURS

either 2,4-di-, 2,3,6-tri-, *o*-, *m*- or *p*-chlorobenzoate. Growth was monitored by measuring O.D. at 510 nm. These data are presented in Figure 9. Because a very distinct chromogenic reaction occurred in the basal salts medium containing sodium benzoate and *m*-chlorobenzoate, the O.D. of the medium did not directly reflect an increase in turbidity due only to the increase in the number of cells. In order to compensate for chromogenesis, O.D. readings were obtained before and after cells were removed by centrifugation.
The O.D. data collected from the growth medium consisting of sodium benzoate and *m*-chlorobenzoate at 12, 24 and 30 h, as presented in Figure 9, were calculated by subtracting the O.D. readings observed after centrifugation from O.D. readings observed before centrifugation.

Growth studies of the cometabolism of *m*-chlorobenzoate

In one set of growth studies, the cometabolic capabilities of *P. fluorescens* were examined. Three basal salts broth media were used and each contained either (1) *m*-chlorobenzoate, (2) *m*-chlorobenzoate plus sodium benzoate or (3) sodium benzoate. In this study, all benzoates were used at a level of 3.5 mmoles per liter of medium. Growth of *P. fluorescens* was assayed by measuring O.D. at 510 nm. Catechol was assayed by the method of Arnow, and the release of chloride ions was detected with a chloride ion-sensing electrode. In addition, chromogenesis was detected by measuring O.D. at 510 nm of clarified media. Chromogenesis only occurred in the medium containing sodium benzoate and *m*-chloro-

Figure 9. The growth of *P. fluorescens* in basal salts broth containing 3.5 mmoles sodium benzoate per liter of medium and supplemented with either 3.5 mmoles 2,4-di- (open squares), 2,3,6-tri- (closed squares), *o*- (open circles), *m*- (closed circles) or *p*-chlorobenzoate (open circles, broken line) per liter of medium. A 1.0 % inoculum of *P. fluorescens* was used to inoculate all media and incubation occurred at 30°C with shaking. Growth was determined by measuring O. D. at 510 nm.


ABSORBANCE 510
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
2
4
6
8
10
12
24
30
TIME IN HOURS

benzoate. These data are presented in Figure 10.

The levels of catechol and chloride ions, as well as growth of *P. fluorescens* in basal salts broth containing 3.5 mmoles sodium benzoate plus 3.5 mmoles *m*-chlorobenzoate per liter of medium are presented in Figure 11. Catechol levels and growth of *P. fluorescens* in basal salts broth medium containing exclusively 3.5 mmoles sodium benzoate per liter are presented in Figure 12. No chloride ions were released into this medium. *Pseudomonas fluorescens* was unable to grow in the medium consisting of basal salts and only *m*-chlorobenzoate. Neither catechol nor chloride ions were detected in this medium.

The capacity of *P. fluorescens* to cometabolize *m*-chlorobenzoate was investigated. Growth of the organism in basal salts media containing 3.5 mmoles glucose and 3.5 mmoles glucose plus 3.5 mmoles *m*-chlorobenzoate per liter of medium was examined by viable cell counts. Catechol was also assayed by the Arnow method. Viable cell numbers of *P. fluorescens* collected from these media are presented in Figure 13. Neither catechol nor chloride ions were detected in any of these media, and chromogenesis did not occur.

Cometabolism of *m*-chlorobenzoate by *P. fluorescens* grown on alternative aromatic carbon sources

The main intent of this experiment was to examine the cometabolic capabilities of *P. fluorescens* grown at the expense of aromatic carbon souces other than sodium benzoate. In basal salts media

Figure 10. Chromogenesis detected in basal salts broth containing 3.5 mmoles *m*-chlorobenzoate plus 3.5 mmoles sodium benzoate. A 1.0 % inoculum was used to inoculate the medium and cultures were incubated at 30°C with shaking. Chromogenesis was determined by measuring the O.D. of the medium after cells were removed by centrifugation.

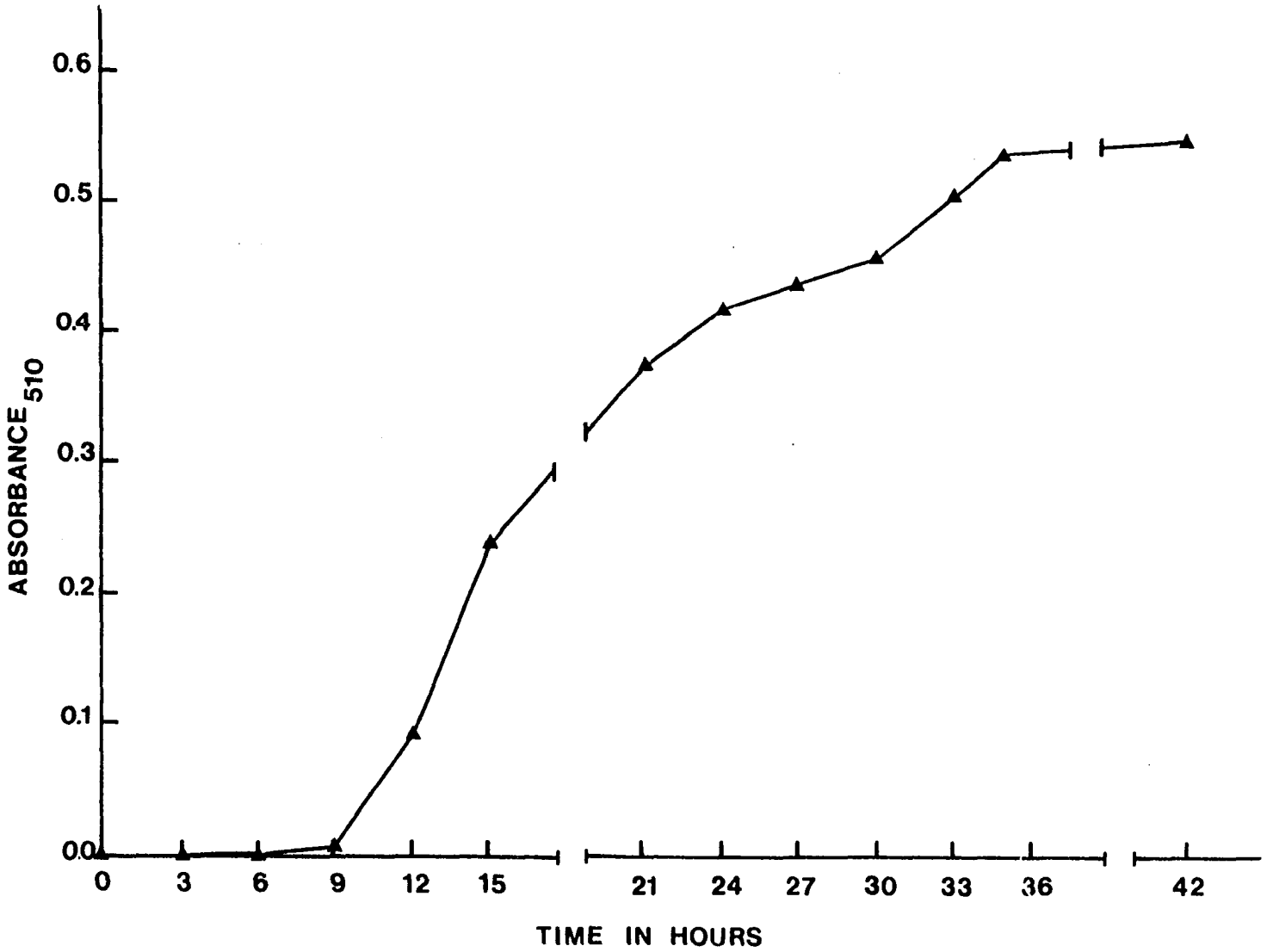

0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE 510
0
3
6
9
12
15
21
24
27
30
33
36
42
TIME IN HOURS

Figure 11. Chloride ion levels (open circles), catechol levels (closed circles) and growth of *P. fluorescens* (open squares) in basal salts broth containing 3.5 mmoles *m*-chlorobenzoate plus 3.5 mmoles sodium benzoate per liter of medium. A 1.0 % inoculum was used to inoculate the medium and the culture was incubated at 30°C with shaking. Chloride ions were detected by using a chloride ion-sensing electrode and catechol was detected via the method of Arnow. Growth was determined by measuring O.D. of the medium at 510 nm.

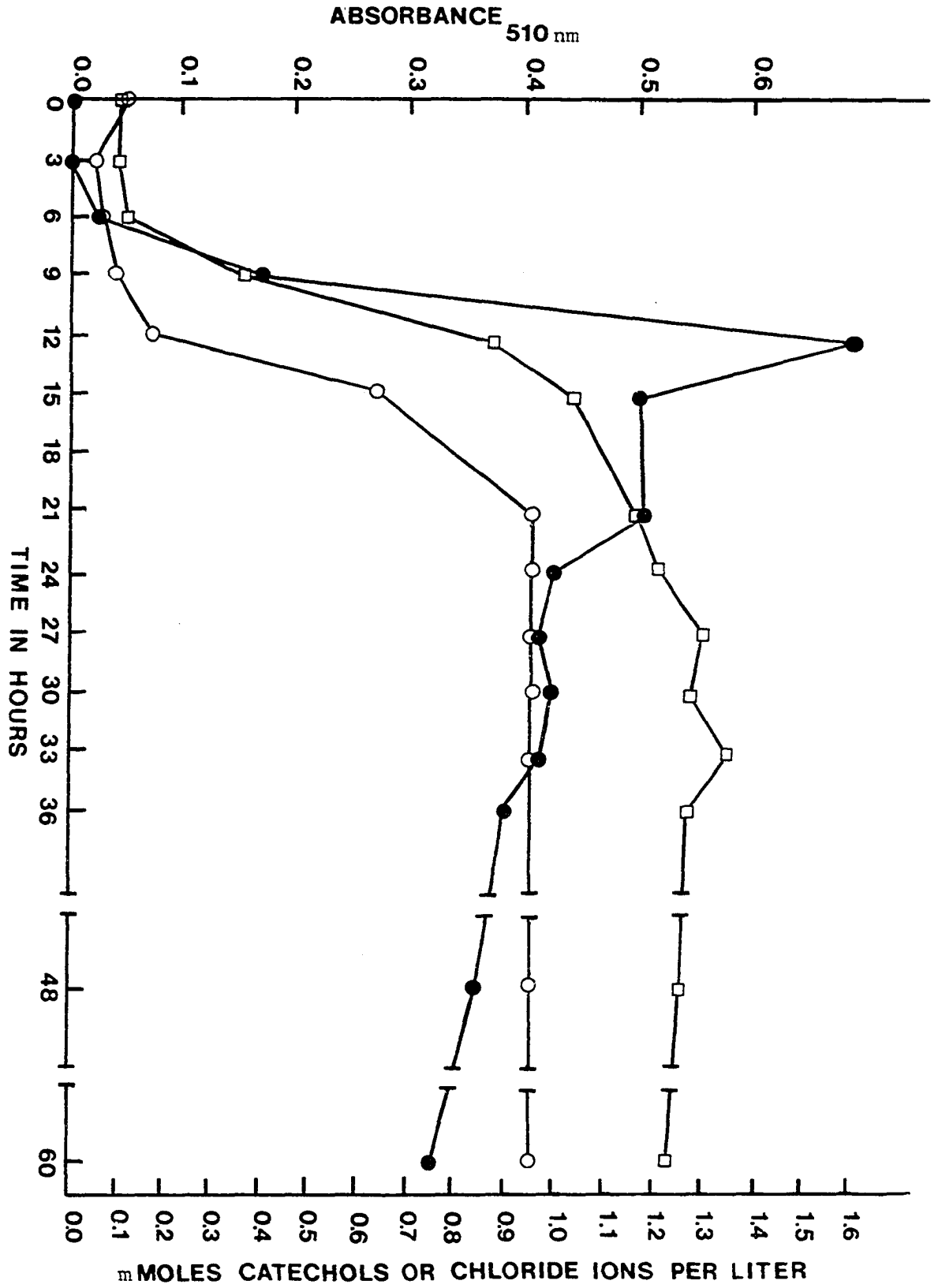

ABSORBANCE 510 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
TIME IN HOURS
0
3
6
9
12
15
18
21
24
27
30
33
36
48
60
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
m MOLES CATECHOLS OR CHLORIDE IONS PER LITER

Figure 12. Catechol levels (circles) and growth of *P. fluorescens* (squares) in basal salts broth medium containing 3.5 mmoles sodium benzoate per liter of medium. A 1.0 % inoculum was used to inoculate the medium and the culture was incubated with shaking at 30°C. Catechol was detected by the method of Arnow and growth was determined by measuring O.D. of the medium at 510 nm.

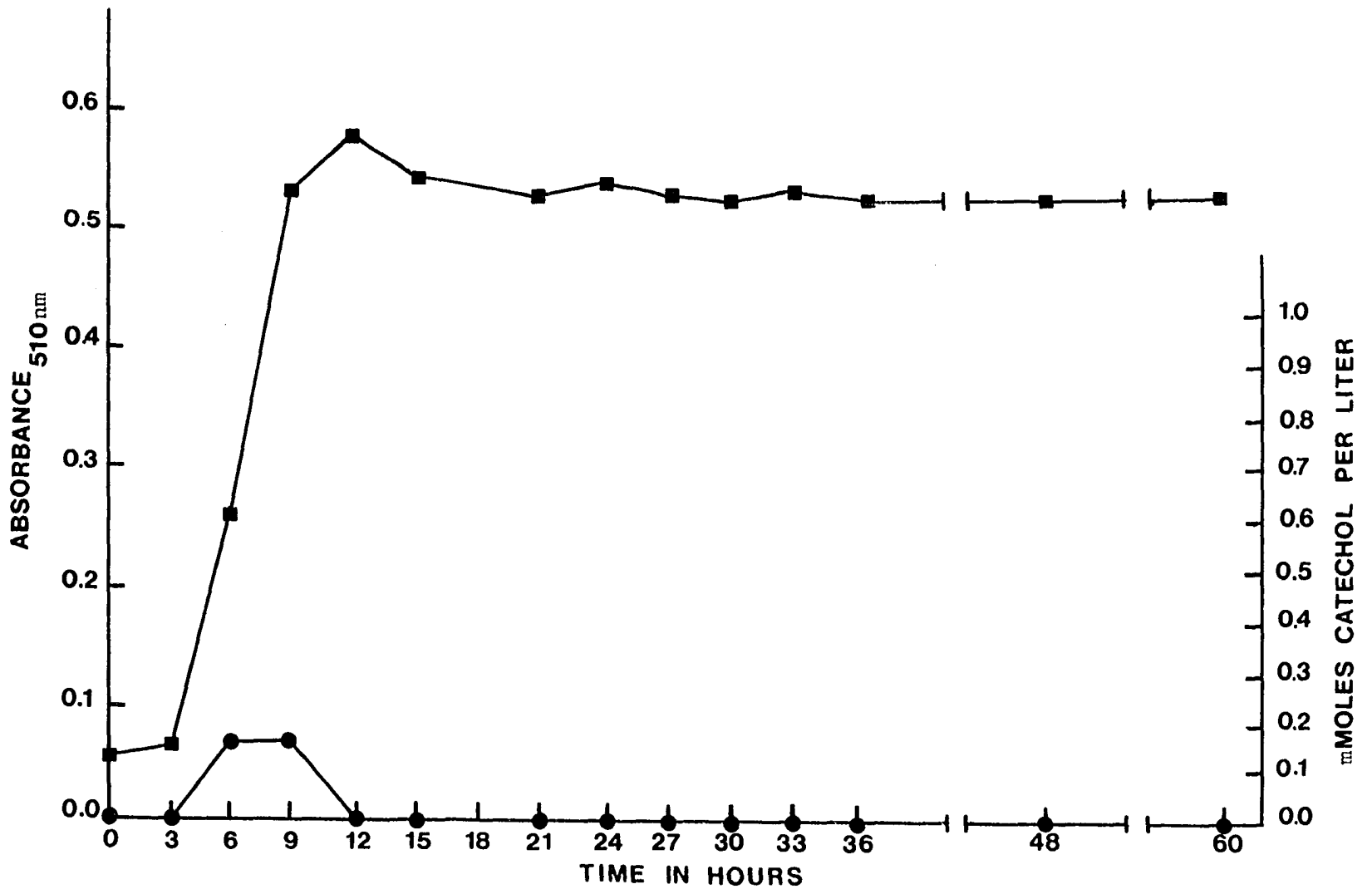
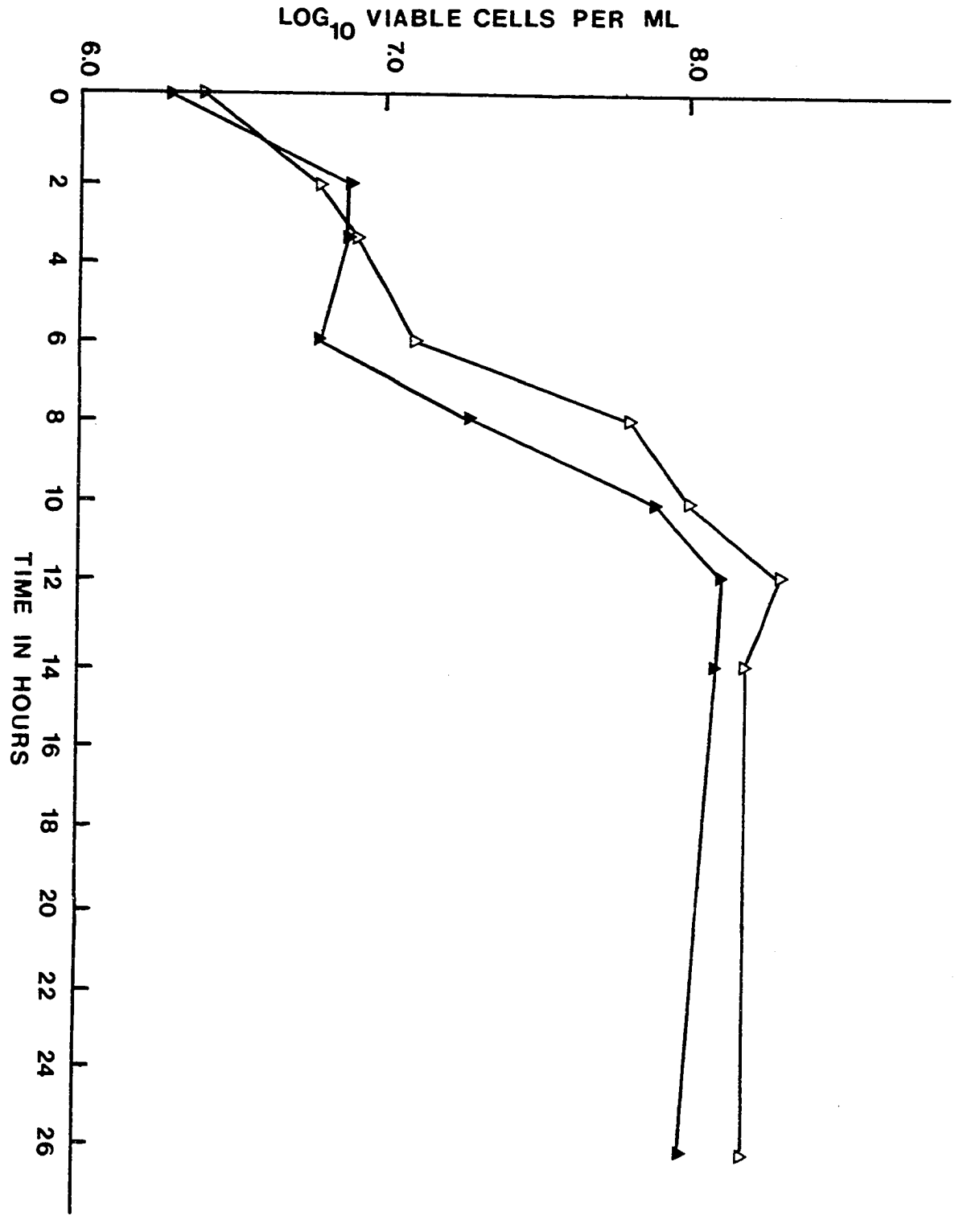

ABSORBANCE 510 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
mMOLES CATECHOL PER LITER
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
3
6
9
12
15
18
21
24
27
30
33
36
48
60
TIME IN HOURS

Figure 13. The growth of *P. fluorescens* in basal salts broth containing 3.5 mmoles glucose (open triangles) or 3.5 mmoles glucose plus 3.5 mmoles *m*-chlorobenzoate (closed triangles) per liter of medium. Media were inoculated with a 1.0 % inoculum of the bacterium and were incubated at 30°C with shaking. Growth was determined by measuring viable cell numbers. Samples of diluted samples were placed in plates containing TSA and the spread-plate technique was used to inoculate all plates. Colonies were counted after at least 24 h of incubation at 30°C.

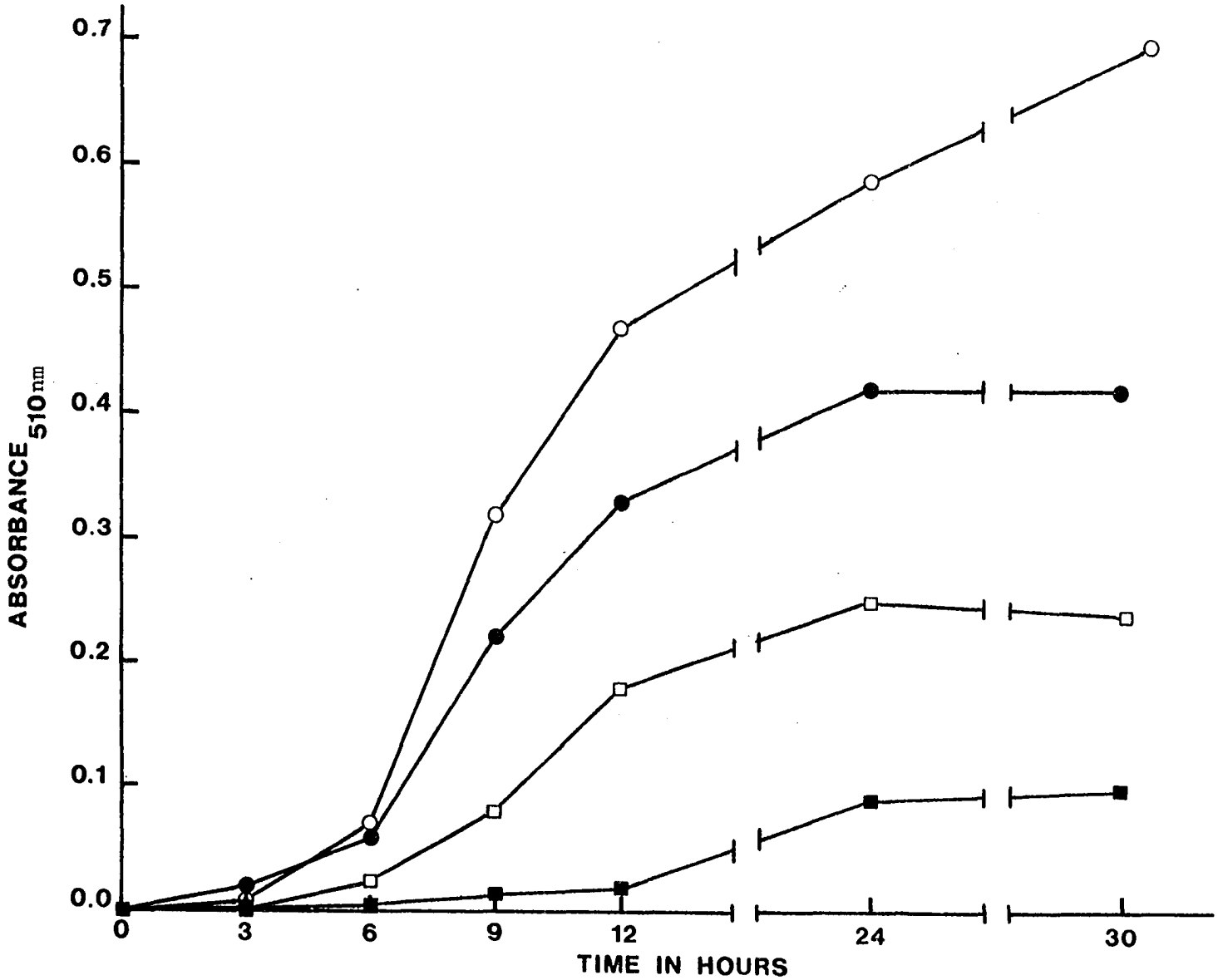

LOG10 VIABLE CELLS PER ML
6.0
7.0
8.0
TIME IN HOURS
0
2
4
6
8
10
12
14
16
18
20
22
24
26

containing either quinic acid, vanillic acid or shikimic acid and supplemented with *m*-chlorobenzoate, the accumulation of a catechol-positive material was noted. After 24 h of growth, 1.6, 1.8 and 1.5 mmoles of catechol-positive material were detected in media containing *m*-chlorobenzoic acid supplemented with quinic acid, vanillic acid or shikimic acid, respectively. All of these media exhibited the distinct purple color similar to chromogenesis observed in the basal salts medium plus sodium benzoate and supplemented with *m*-chlorobenzoate. Neither purple color nor accumulated catechols were observed in media containing quinic acid, vanillic acid or shikimic acid devoid of *m*-chlorobenzoate.

Growth studies of *P. fluorescens* during the cometabolism of *m*-chlorobenzoate at various levels of sodium benzoate

Eight modified basal salts broth media were used in this study. Four media contained 3.5 mmoles *m*-chlorobenzoate per liter, whereas the remaining four media were used as controls and contained no chlorobenzoate. Sodium benzoate was added to the control and chlorobenzoate-containing media at four different levels: 3.5, 2.624, 1.75 or 0.875 mmoles per liter. The purpose of this study was to identify what influence the level of substrate had on cometabolic properties, including catechol production, chromogenesis and halide release. Growth of *P. fluorescens* in these media was measured by monitoring O.D. at 510 nm. Growth data for media containing both sodium benzoate and *m*-chlorobenzoate are presented in Figure 14.

Figure 14. The growth of *P. fluorescens* in basal salts broth containing 3.5 mmoles *m*-chlorobenzoate and 3.5 (open circles), 2.625 (closed circles), 1.75 (open squares) or 0.875 (closed squares) mmoles sodium benzoate per liter of medium. All media were inoculated with a 1.0 % inoculum of the bacterium and cultures were incubated at 30°C with shaking. Growth was determined by monitoring O.D. at 510 nm.


ABSORBANCE 510nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
3
6
9
12
24
30
TIME IN HOURS

Growth data for media containing only sodium benzoate are presented in Figure 15. Chromogenesis of clarified media was detected only in media supplemented with *m*-chlorobenzoate, and these results are presented in Figure 16. Chloride ions were released into media containing both sodium benzoate and *m*-chlorobenzoate and these data appear in Figure 17. Catechol was detected via the Arnow method in the media containing sodium benzoate plus *m*-chlorobenzoate, and these data are presented in Figure 18. In the four media containing only sodium benzoate, catechol was detected only at 6 h during the growth of the organism. Less than 0.1 mmole catechol per liter was noted in all four media at 6 h. At 9 h, however, 0.38 mmole of catechol was detected in the medium containing 3.5 mmoles sodium benzoate per liter. No catechol was detected in this medium after incubation for 12 h.

Growth of *P. fluorescens* during cometabolism of *m*-chlorobenzoate in the absence and presence of iron

The objective of this study was to examine the effects that iron had on growth of *P. fluorescens*, catechol production, chloride ion release and chromogenesis. Two media were employed in this study. Both contained 3.5 mmoles of *m*-chlorobenzoate and 3.5 mmoles sodium benzoate per liter of basal salts broth; in one medium the $Fe(NH_4)(SO_4)\cdot 12H_2O$ was omitted from the basal salts broth and in the other the iron salt was included. Growth was determined by measuring O.D. at 510 nm before and after centrifugation of the media. The O.D. at 510 nm determined after centrifugation served as a measure of

Figure 15. The growth of *P. fluorescens* in basal salts broth containing either 3.5 (open circles), 2.625 (closed circles), 1.75 (open squares), or 0.875 (closed squares) mmoles sodium benzoate per liter. Media were inoculated with a 1.0 % inoculum of the bacterium and cultures were incubated at 30°C with shaking. Growth was determined by monitoring O.D. at 510 nm.

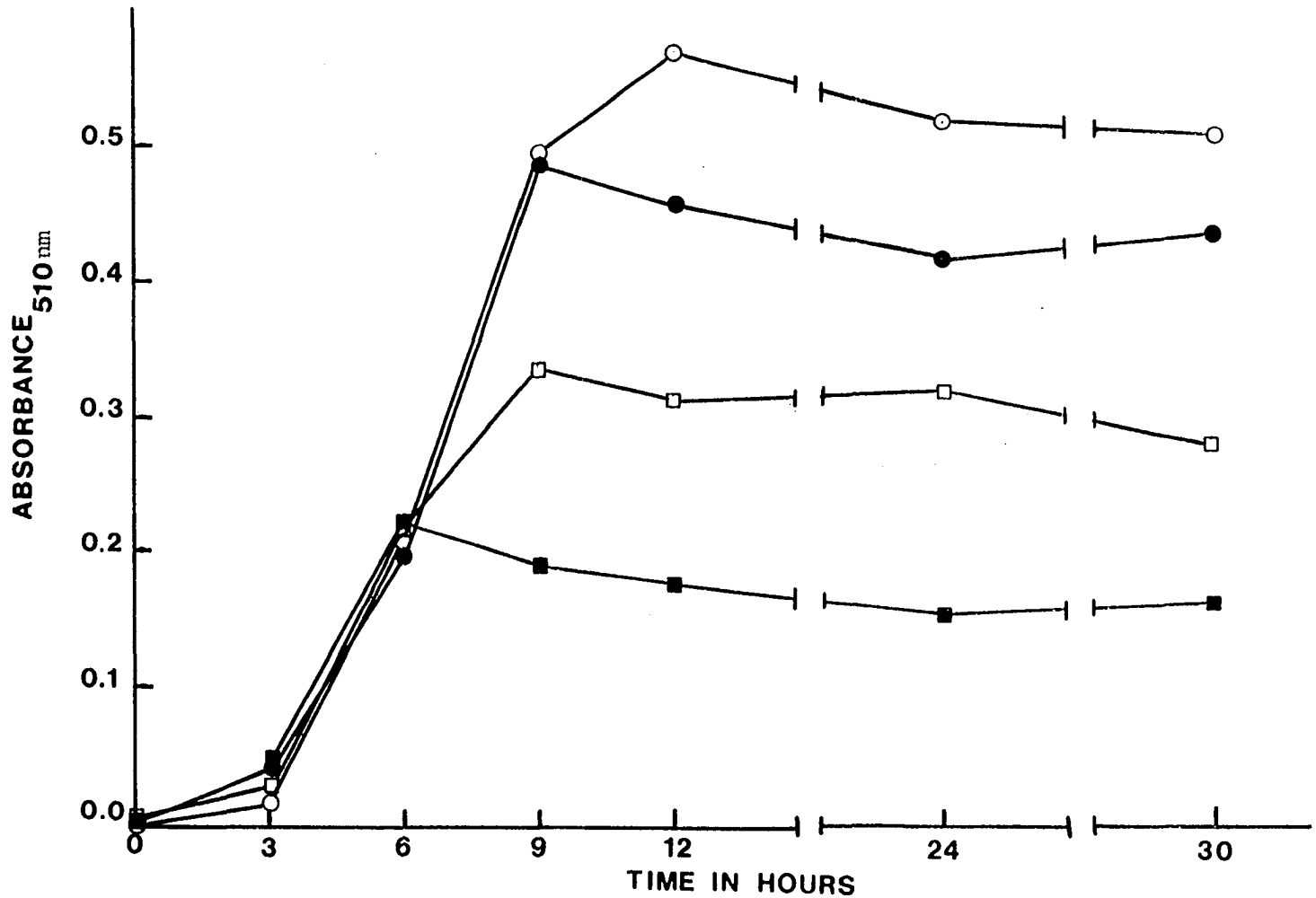

ABSORBANCE 510 nm
0.5
0.4
0.3
0.2
0.1
0.0
0
3
6
9
12
24
30
TIME IN HOURS

Figure 16. Chromogenesis detected in basal salts broth containing 3.5 mmoles *m*-chlorobenzoate and supplemented with either 3.5 (open circles), 2.625 (closed circles), 1.75 (open squares) or 0.875 (closed squares) mmoles of sodium benzoate per liter. Media were inoculated with a 1.0 % inoculum of actively growing *P. fluorescens* and cultures were incubated at 30°C with shaking. Chromogenesis was measured by monitoring O.D. of centrifuged media at 510 nm.

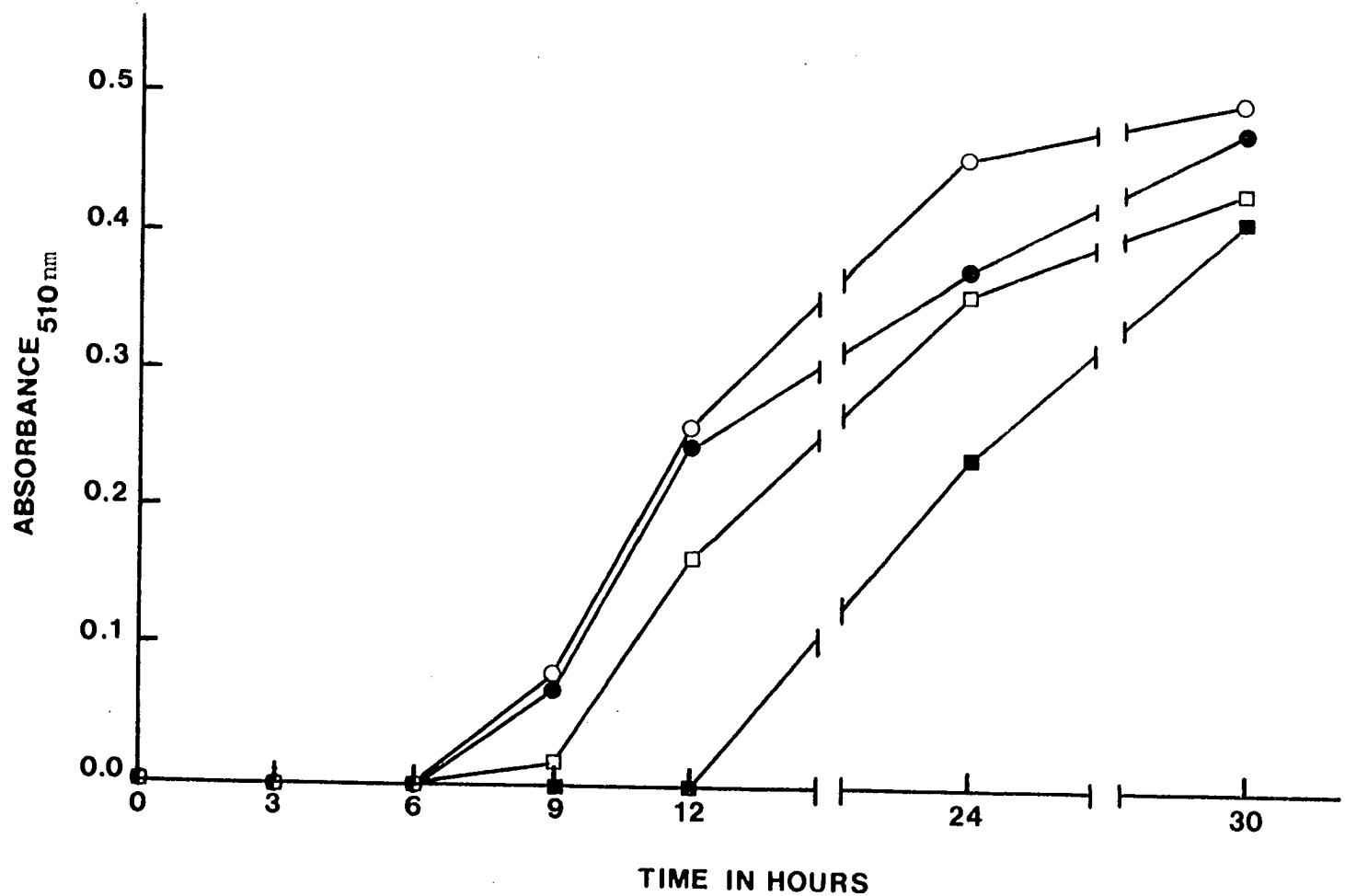

ABSORBANCE 510 nm
0.5
0.4
0.3
0.2
0.1
0.0
0
3
6
9
12
24
30
TIME IN HOURS

Figure 17. Chloride ions detected in basal salts broth containing 3.5 mmoles *m*-chlorobenzoate and supplemented with either 3.5 (open circles), 2.625 (closed circles), 1.75 (open squares) or 0.875 (closed squares) mmoles of sodium benzoate per liter. Media were inoculated with a 1.0 % inoculum of actively growing bacteria and cultures were incubated at 30°C with shaking. Chloride ion release was determined with a chloride ion-sensing electrode.

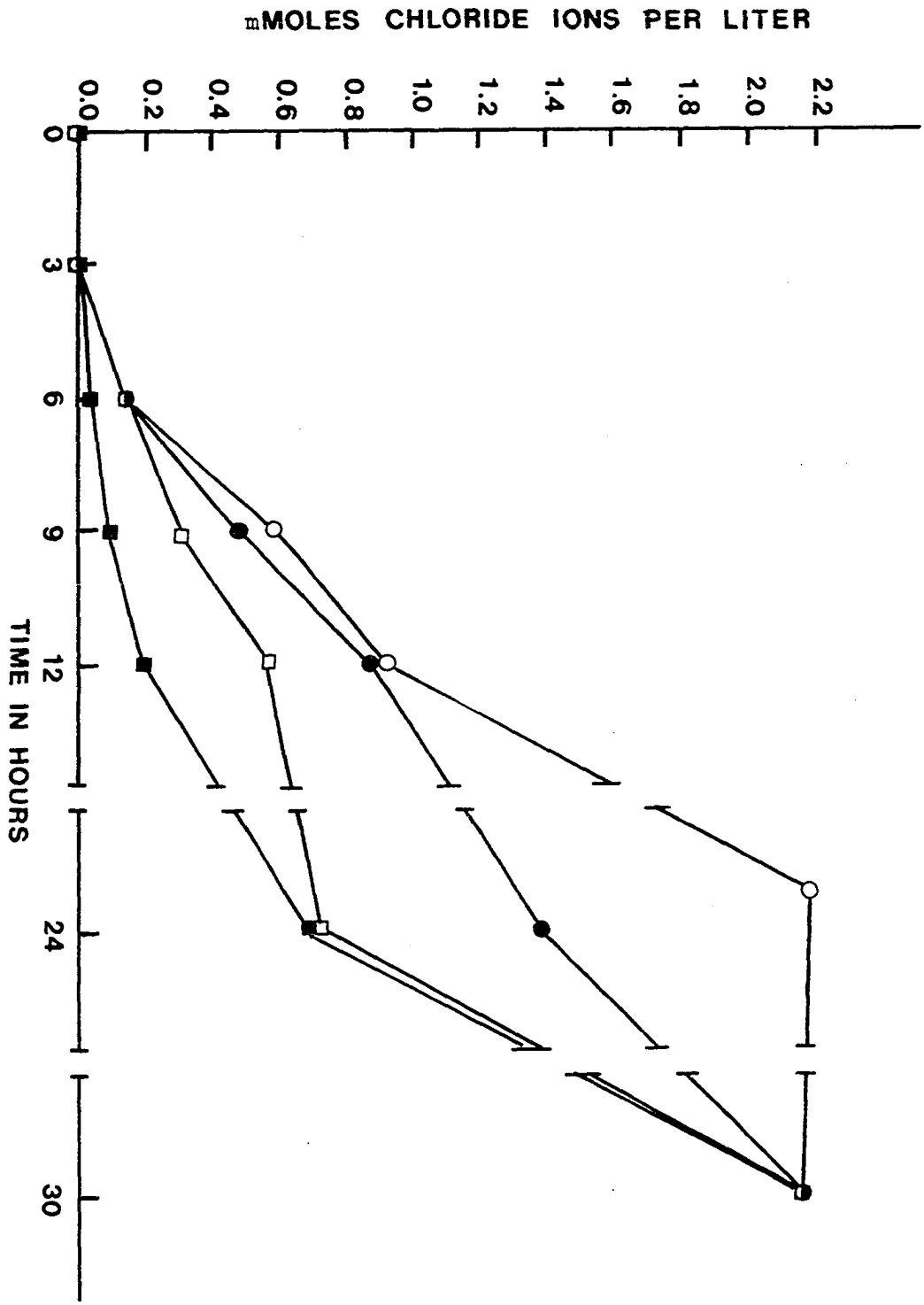
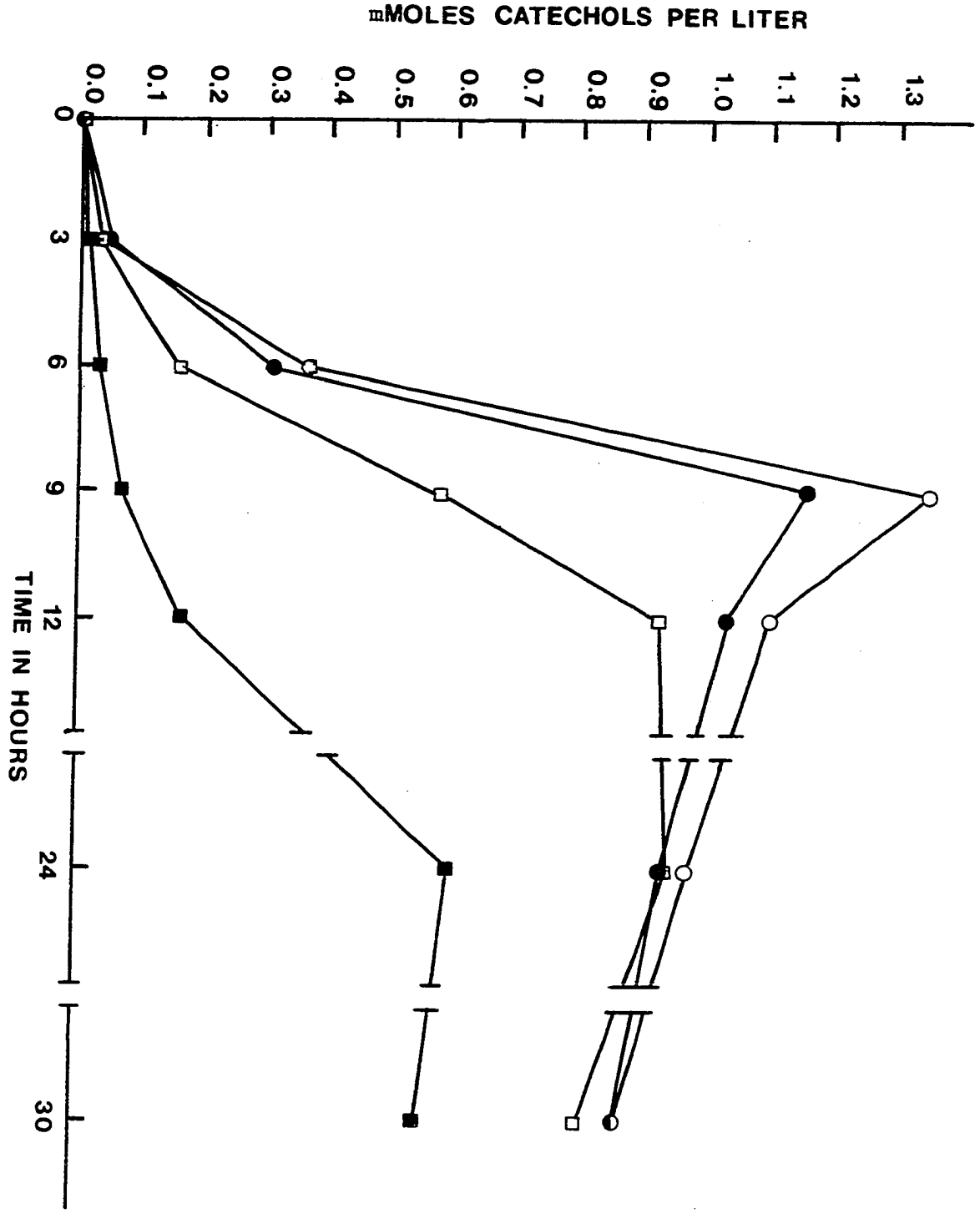

mMOLES CHLORIDE IONS PER LITER
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
TIME IN HOURS
0
3
6
9
12
24
30

Figure 18. Catechol levels in basal salts broth containing 3.5 mmoles *m*-chlorobenzoate and 3.5 (open circles), 2.625 (closed circles), 1.75 (open squares) or 0.875 (closed squares) mmoles sodium benzoate per liter of medium. Media were inoculated with a 1.0 % inoculum of actively growing *P. fluorescens* and cultures were incubated at 30°C with shaking. Catechol was detected via the Arnow method.


mMOLES CATECHOLS PER LITER
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
1.3
TIME IN HOURS
0
3
6
9
12
24
30

chromogenesis. Catechol and chloride ion release were also measured. Growth was not altered by omitting iron from the medium and viable cell numbers observed in the iron-free medium corresponded to those observed in the medium containing iron (data not shown). In addition, the levels of catechol or chloride ions noted in the medium containing no iron were not different from the levels detected in media containing iron. Only chromogenesis was affected by the levels of iron in the medium. When iron was present, chromogenesis occurred as shown in Figure 10. But in the iron-free medium, chromogenesis did not occur until 16 h and was very slight (O.D. 0.04, 510 nm). At 19 h, maximum chromogenesis was noted and was also slight (O.D. 0.07, 510 nm); increased chromogenesis was not observed over a subsequent two-day period.

Growth of *P. fluorescens* in media containing sodium benzoate and supplemented with catechol or 3-chlorocatechol

The effect of various concentrations of catechol on the metabolism of *P. fluorescens* grown on sodium benzoate was investigated by supplementing basal salts media containing 3.5 mmoles sodium benzoate per liter with either 0.25, 0.5, or 2.0 mmoles of catechol per liter of medium. After a 1.0 % inoculum of *P.fluorescens* was added, incubation progressed at 30°C with shaking. During this period, samples were collected to assay growth (by measuring O.D. at 510 nm) and to determine catechol levels (via the method of Arnow). The growth of *P. fluorescens* in all four of these

media is presented in Figure 19 and catechol levels detected in these growth media are shown in Figure 20. No catechol was detected after 12 h of incubation which suggested that all of the catechol initially present in the medium was utilized for the growth of *P. fluorescens*.

When 3-chlorocatechol was supplied to *P. fluorescens* as a sole source of carbon and energy at levels of 3.5 and 1.75 mmoles per liter of basal salts broth, the organism was unable to grow. In fact, no viable cells were recovered from the medium supplemented with 3.5 mM 3-chlorocatechol after 10 h of inoculation. In the medium containing 1.75 mM 3-chlorocatechol, all cells were dead within 48 h after inoculation. Cells maintained viability for at least 48 h, however, in basal salts medium containing no carbon source. An initial concentration of 1.0×10^7 cells per ml was detected in basal salts medium and 1.0×10^6 viable cells remained after 48 h of incubation. Alternatively, when cells were placed in basal salts broth medium containing 3.5 or 1.75 mmoles catechol per liter, *P. fluorescens* grew well and viable cell data from this study appear in Figure 21. The purple color of the medium disappeared from the medium containing 1.75 mM catechol by 10 h. The purple color remained in the medium containing 3.5 mM catechol through 24 h but disappeared by 48 h of incubation.

By 10 h of incubation, no catechol was detectable in the medium initially containing 1.75 mM catechol, but 0.7 mM of catechol remained in basal salts broth initially supplemented with 3.5 mM catechol,

Figure 19. Growth of *P. fluorescens* in basal salts broth containing 3.5 mmoles sodium benzoate per liter of medium and supplemented with either 0 (open circles), 0.25 (closed circles), 0.5 (closed squares) or 2.0 (open squares) mmoles catechol per liter of medium. A 1.0 % inoculum was used to inoculate all media and incubation occurred at 30°C with shaking. Growth was monitored by measuring viable cell numbers.

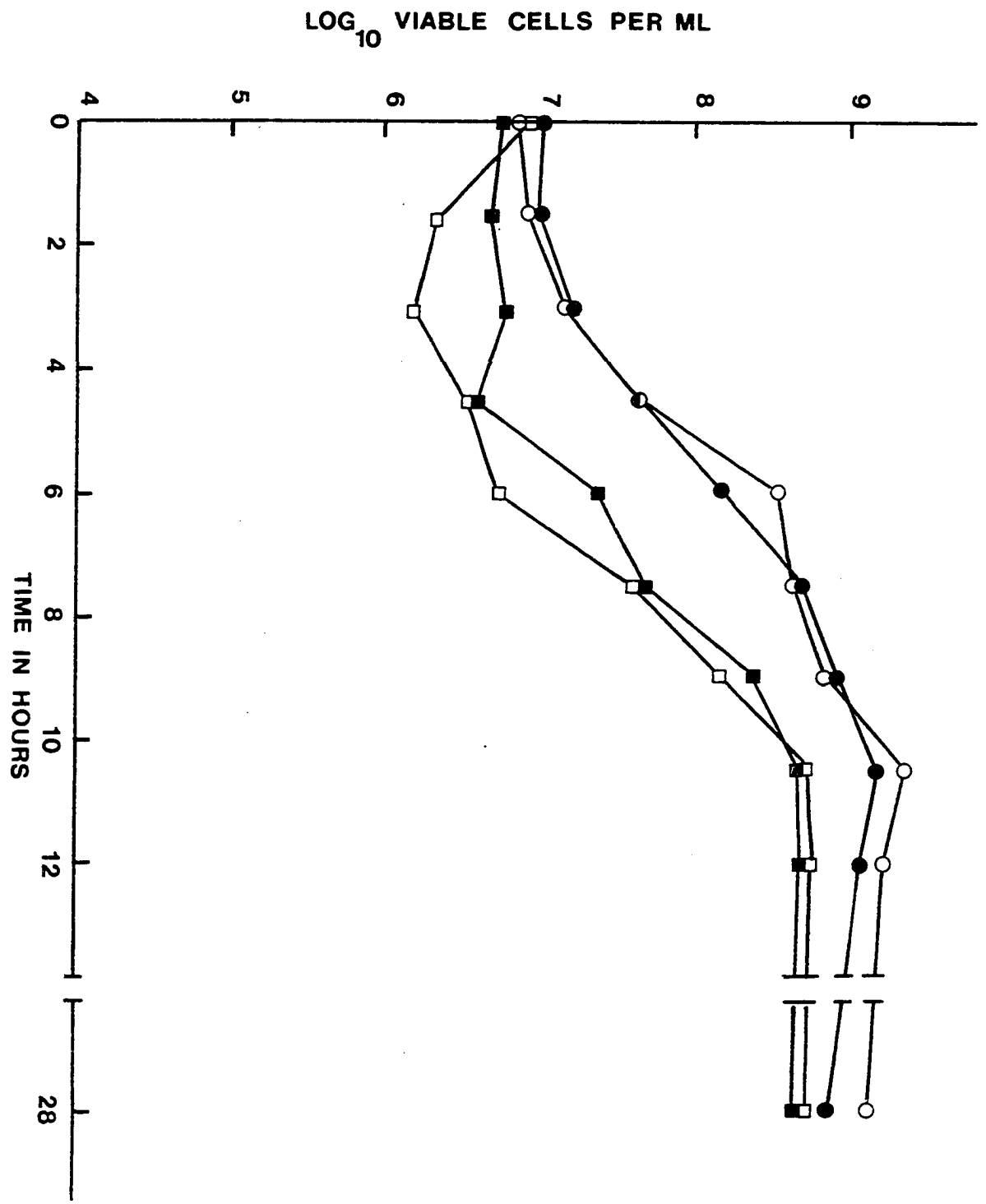

LOG10 VIABLE CELLS PER ML
4
5
6
7
8
9
TIME IN HOURS
0
2
4
6
8
10
12
28

Figure 20. Catechol levels in basal salts broth containing 3.5 mmoles sodium benzoate per liter of medium and supplemented with either 0 (open circles), 0.25 (closed circles), 0.5 (closed squares) or 2.0 (open squares) mmoles catechol per liter of medium. A 1.0 % inoculum was used to inoculate all media, and incubation was at 30^{o}C with shaking. Catechol levels were determined via the method of Arnow.

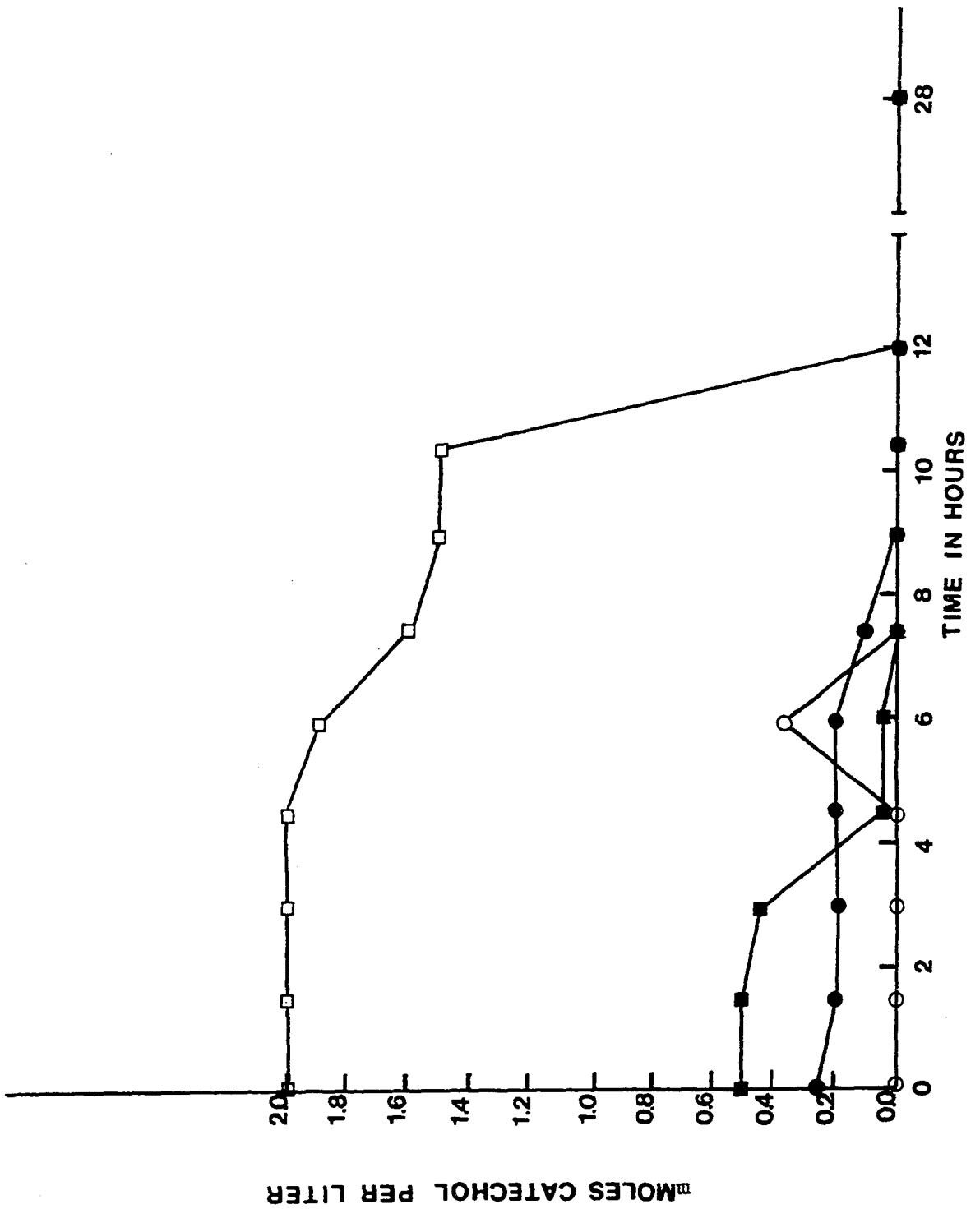

μMOLES CATECHOL PER LITER
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
28
TIME IN HOURS

Figure 21. The growth of *P. fluorescens* in basal salts broth media containing 3.5 (open triangles) or 1.75 (closed triangles) mmoles catechol per liter of medium. Media were inoculated with a 1.0 % inoculum and were incubated at 30°C with shaking. Samples were collected and growth was measured by placing samples or diluted samples in petri plates containing TSA. Plates were inoculated via the spread-plate technique and were incubated for at least 24 h before colonies were counted.

LOG10 VIABLE CELLS PER ML
4
5
6
7
8
9
TIME IN HOURS
0
2
4
6
8
10
24
48

even after 48 h of incubation.

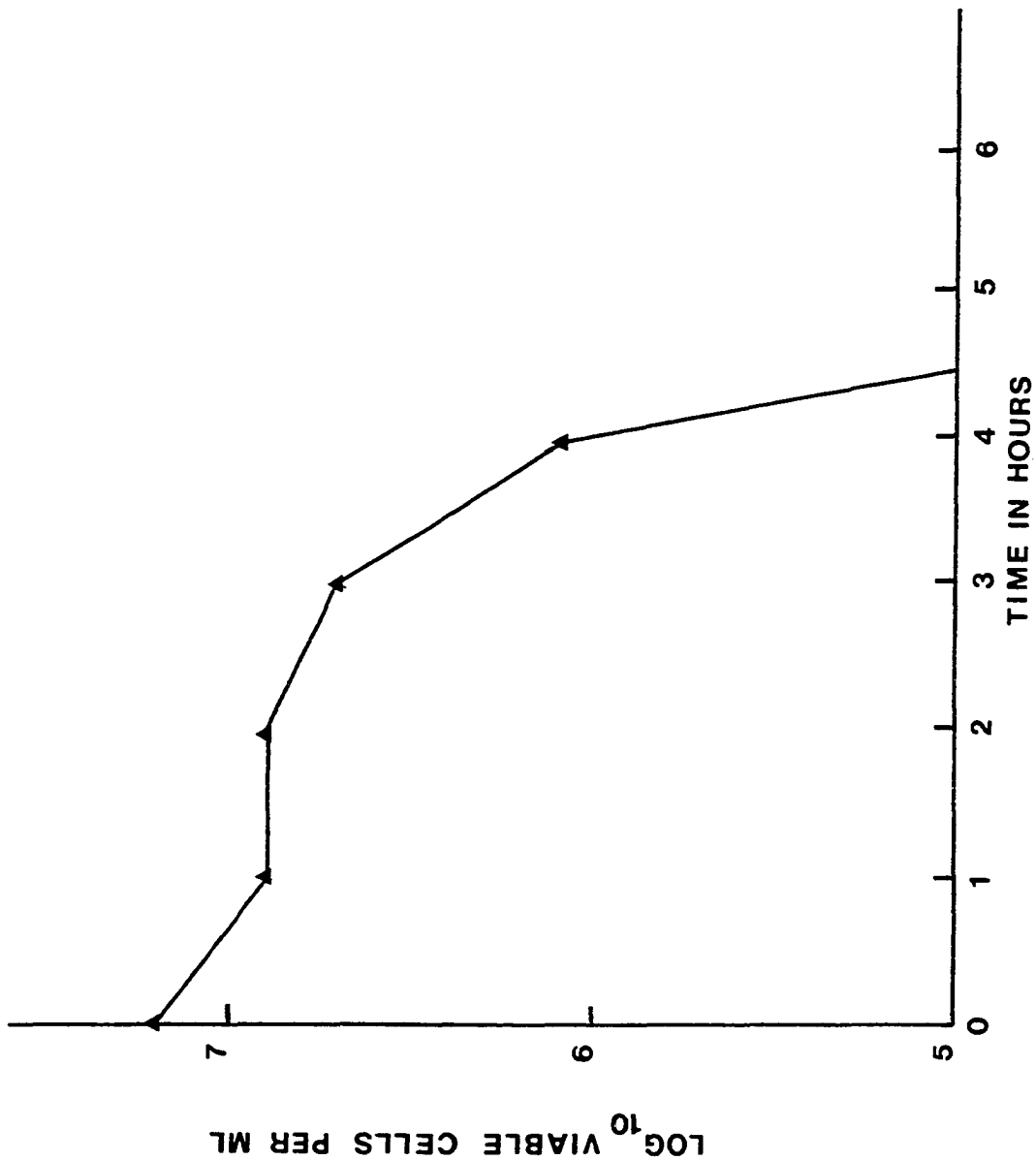
In one additional study which was performed to determine whether or not *P. fluorescens* could mineralize 3-chlorocatechol, a medium containing 3.5 mM sodium benzoate was used. The chlorocatechol was added sequentially to this basal salts broth medium at 2, 4, 6, 8 and 10 h during growth. Viable cell numbers, catechol levels and chloride ion levels were monitored. Five 0.7-mmole portions were added to this medium at 2 h intervals, resulting in final concentrations of 0.7, 1.4, 2.1, 2.8 and 3.5 mmoles of chlorocatechol per liter of medium. Each portion was added immediately after samples were collected at 2, 4, 6, 8 and 10 h.

No chloride ions were released into the medium and 3-chlorocatechol was not utilized. Viable cells were monitored by plating samples on TSA, and at 5 h, no viable cells remained in the medium (Figure 22).

Resting cell studies

3-Chlorocatechol was detected in resting cell media containing 3.5 or 1.75 mmoles of *m*-chlorobenzoate per liter of basal salts. Cells were prepared by harvesting cells of *P. fluorescens* from a growth medium containing 3.5 mmoles of sodium benzoate and 3.5 mmoles of *m*-chlorobenzoate per liter of medium. Cells were harvested at 3, 6, 9 and 12 h and were added to resting cell media as previously described. No significant differences in catechol production occurred in media incubated with cells harvested at 3, 6 or 9 h

Figure 22. Viable cells of *P. fluorescens* in a basal salts broth medium containing 3.5 mmoles sodium benzoate per liter and sequentially-supplemented with 3-chlorocatechol. A total of five 0.7-mmole portions of 3-chlorocatechol were added to this medium in sequence at 2 h intervals, to result in final concentrations of 0.7, 1.4, 2.1, 2.8 and 3.5 mmoles per liter of medium. Each portion was added immediately after samples were collected at 2,4,6,8 and 10 h. At 5 h no viable cells remained in the medium. Viable cell numbers were determined by placing samples or diluted samples in petri plates containing TSA. Plates were inoculated via the spread-plate technique and were incubated for at least 24 h before colonies were counted.

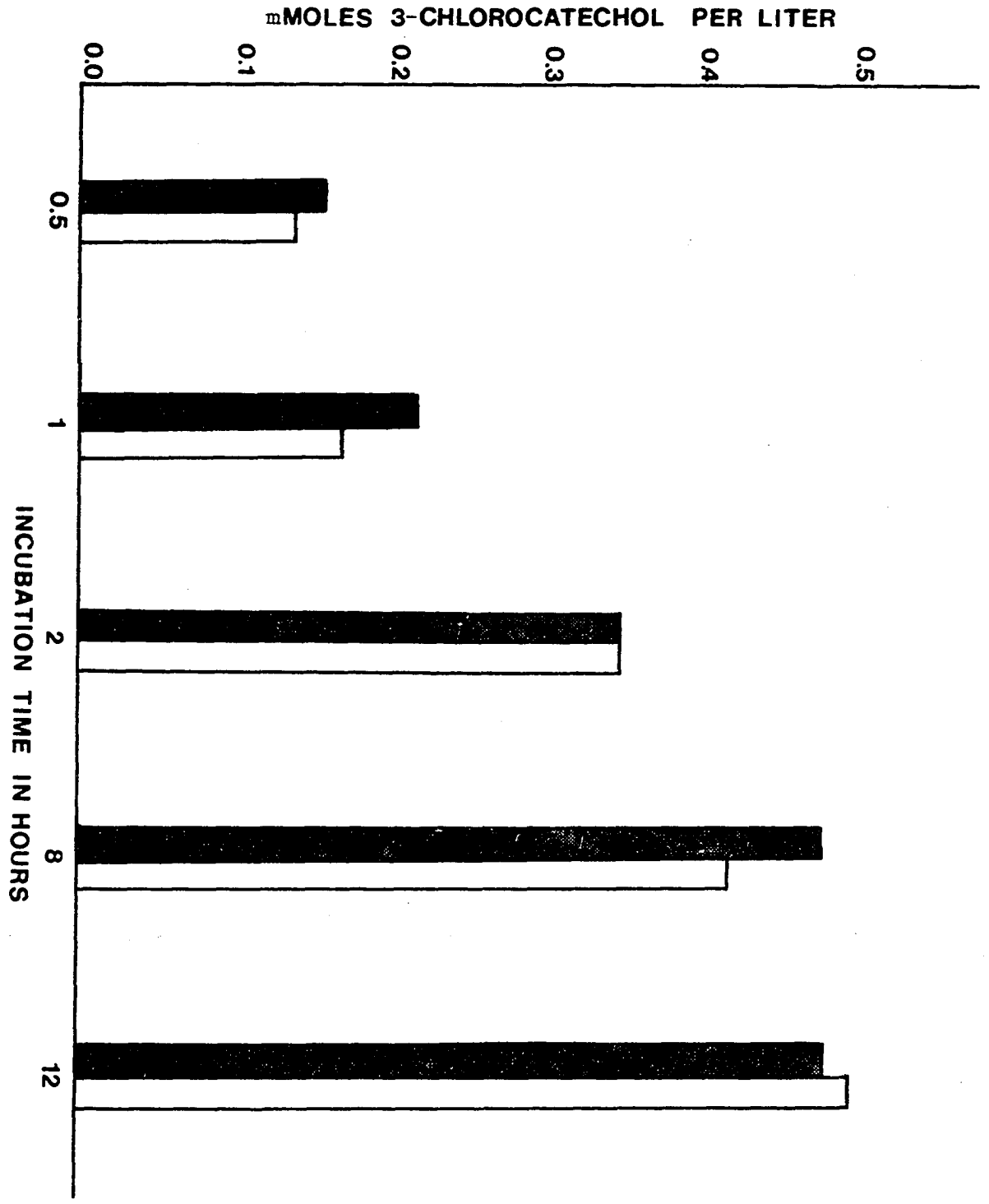

LOG10 VIABLE CELLS PER ML
7
6
5
0
1
2
3
4
5
6
TIME IN HOURS

during the growth curve. The amounts of catechol increased during resting cell incubation and approximately 0.5 mmoles of 3-chlorocatechol accumulated in all resting cell media. All reaction media were purple after 12 h of incubation. Neither the presence of NADH nor the concentration of *m*-chlorobenzoate affected overall accumulation, but catechol accumulated most rapidly in reactions containing 3.5 mmoles of *m*-chlorobenzoate. No catechol was detected in resting cell media incubated with cells that were harvested at 12 h during growth. The catechol levels detected in resting cell media containing either 2.5 mM or 1.75 mM *m*-chlorobenzoate and inoculated with cells harvested at 3 h appear in Figure 23.

Chromogenesis disappearance study

This experiment was performed to examine the details of purple utilization and to determine if low levels of purple color would disappear in the presence of actively-growing cultures. In this study, 3-chlorocatechol was allowed to become purple by reacting with ferric ions. The chromogenic 3-chlorocatechol was added at levels of either 0.21 or 0.035 mmoles per liter of broth media in which *P. fluorescens* was actively growing at the expense of either glucose, benzoate, protocatechuate, fumarate or catechol. These cultures had been allowed to grow to the mid-log phase of growth in broth media containing 3.5 mmmoles of each substrate. These media were incubated with shaking at room temperature. Initially, all media were purple after the addition of 3-chlorocatechol,

Figure 23. The production of 3-chlorocatechol in resting cell media. Basal salts medium supplemented with either 3.5 (dark bars) or 1.75 (light bars) mmoles *m*-chlorobenzoate per liter were inoculated with cells harvested from a growth medium. This medium consisted of basal salts medium supplemented with 3.5 mmoles sodium benzoate and 3.5 mmoles *m*-chlorobenzoate per liter. Cells were harvested at 3 h of growth, centrifuged and resuspended in basal salts. The O.D. of the suspension was adjusted to 0.2 and 0.5 ml were added to each of various tubes containing 4.5 ml of resting cell medium. Catechol was assayed at 0.5, 1, 2, 8 and 12 h via the Arnow method.


mMOLES 3-CHLOROCATECHOL PER LITER
0.0
0.1
0.2
0.3
0.4
0.5
INCUBATION TIME IN HOURS
0.5
1
2
8
12

and uninoculated controls containing 3-chlorocatechol were used as color references.

At 8 h after the addition of 3-chlorocatechol, all media were purple and the benzoate medium supplemented with the higher levels of 3-chlorocatechol was intensely purple. By 18 h, the purple color of this medium had resumed to its original intensity.

After 4 days, it was difficult to observe purple color in any media supplemented with the lower level of 3-chlorocatechol. All media supplemented with 0.21 mmoles of 3-chlorocatechol were no longer purple, but they possessed a grey hue and a black precipitate had formed in these media. The pH of the media after 4 days of incubation was 7.0. The color references remained purple at this time, but the intensity of color did not seem as great as initially observed.

Color reactions and ultraviolet absorption maxima of catechols extracted from cometabolic growth media and resting cell media

In one experiment designed to investigate color reactions of catechols, basal salts medium was prepared and supplemented with either catechol, 3-chlorocatechol, 4-chlorocatechol, catechols extracted from cometabolic growth medium that contained sodium benzoate and *m*-chlorobenzoate and a catechol extracted from resting cell media containing *m*-chlorobenzoate. The catechols were each added to basal salts medium and were incubated with shaking at room temperature for 24 h. The pH of each mixture was then adjusted to either 1.5, 6.0, 7.0, 8.0 or 9.0. The colors of these media were then recorded

following visual examination and the results are summarized in Table 3.

All of the media supplemented with catechols were examined via ultraviolet spectroscopy and absorption maxima were recorded. The results of these examinations appear in Table 4. After one week, each medium was examined for the presence of insoluble precipitates. All of the media exhibited a dark precipitate after this period of time.

To determine whether or not any particular constituent of the basal salts medium was responsible for chromogenesis, each salt used in the medium was examined. Salts were used independently and in every possible combination to determine if any particular constituent of the medium was responsible for purple color production. An iron influence on chromogenesis was indicated in previous growth studies. It was discovered in this study that when $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ and K_2HPO_4 were both present in any medium, purple color was produced. When only $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ was present in the medium, the addition of 3-chlorocatechol resulted in an olive-green chromogenic reaction. When the pH of this medium was raised by adding 0.1 N NaOH, the olive-green color changed to a purple color. All media became colorless upon the addition of 0.5 N HCl. The pH that resulted from the addition of this acid was generally less than 1.0 and the purple color could be regained upon the addition of NaOH.

Table 3. Color reactions of catechols in basal salts broth. The catechols were added to the basal salts medium to result in a final concentration of 25 ug/ml. All mixtures were allowed to shake at room temperature for 24 h until intense color reactions were noted. The pH of each mixture was adjusted and colors were noted.

	color				
	pH				
supplement	1.5	6.0	7.0	8.0	9.0
catechol	yellow	lavender	violet	purple	dark purple
4-chlorocatechol	yellow	violet	bright violet	pink	pink-orange
3-chlorocatechol	yellow	violet	bright violet	pink	pink-orange
extract from cometabolic growth media	yellow	lavender	violet	purple	dark purple
extract from resting cell medium	yellow	violet	bright violet	pink	pink

Table 4. Ultraviolet absorption maxima of catechols in basal salts broth. Catechols were added to the basal salts broth at a concentration of 25 ug/ml. All mixtures were allowed to shake at room temperature for 24 h until intense color reactions in media were noted. The pH of each medium was adjusted and then each medium was diluted 1:10 in distilled and deionized water. An ultraviolet spectrum (320nm to 220nm) of each medium at each pH level was obtained with a Beckman DB spectrophotometer.

	absorption maxima				
	pH				
supplement	1.5	6.0	7.0	8.0	9.0
	nm	nm	nm	nm	nm
catechol	274	274-275	274-275	274-276	277-279
4-chlorocatechol	282	282-283	282	283-286	290-293
3-chlorocatechol	280	279	280.5	281-282	288-290
extract from cometabolic growth medium	279-281.5	275-278	276-278	276-282	275-285
extract from resting cell medium	279-280	277-278	278-280	279-280	280-281

Detection of *o*-benzoquinones

The detection of *o*-benzoquinones was demonstrated in media containing sodium benzoate and *m*-chlorobenzoate. After adding approximately 1.0 ml aniline per 100 ml medium, flasks containing spent media were incubated without shaking for one week. During this time, crystals were deposited around the sides of the flasks. These were removed and washed with diethyl-ether and red crystals were obtained. The melting point of these crystals was approximately 193°C. A copious amount of metallic crystals which were not soluble in ether were also obtained from flasks containing spent media and the melting point of this material was approximately 210°C.

Identification of accumulated catechols from growth media and resting cell media

Infrared spectra of lead catecholates prepared from catechols isolated from growth media and resting cell media were compared to spectra of lead catecholates prepared from authentic catechol, 4-chlorocatechol, and 3-chlorocatechol. In all spectra, peaks were observed that indicated that presence of aromatic C-O stretch vibrations (1180 and 1330 nm) and aromatic C-C stretch vibrations (1447, 1492, 1610 and 1660 nm). The lead catecholates prepared from catechols extracted from cometabolic growth media and resting cell media with *m*-chlorobenzoate absorbed strongly at 735 and 755 nm. These bands were also noted in infrared spectra of authentic 3-chlorocatechol

samples and these bands were only consistent with 1, 2, 3-aromatic substitution patterns.

To substantiate that the extracted catechols were actually 1,2,3-substituted rather than 1,2,4-substituted, infrared spectra of the natural product were compared to infrared spectra of 4-chlorocatechol and the lead salt of 4-chlorocatechol. The spectra of the 4-chlorocatechol and the lead salt of 4-chlorocatechol absorbed at 789 and 850 nm. These bands were consistent with 1,2,4-substituted aromatic compounds. The lack of bands at 789 and 850 nm in spectra of the catechol isolated from media and resting cell media ruled out 1,2,4-substitution.

The NMR spectra of extracted 3-chlorocatechol from growth media and resting cell media were: (D_6-acetone); δ=6.75 (multiplet, 3 H's, aromatic H's); δ=8.05 (broad singlet, 2 H's, OH). The addition of D_2O caused the singlet at δ=8.05 to disappear, confirming that these protons were hydroxylic. These NMR spectra were consistent with NMR spectra of authentic 3-chlorocatechol.

DISCUSSION

A chromogenic reaction that occurred during a cometabolic conversion was investigated in this study. Cometabolism was implicated because *m*-chlorobenzoate could not support the growth of *Pseudomonas fluorescens*. The bacterium grew, however, at the expense of sodium benzoate, a biodegradable analog of *m*-chlorobenzoate. Seven mmoles of sodium benzoate per liter of basal salts medium supported more growth than 3.5 mmoles of sodium benzoate per liter of basal salts (Figure 7). The growth of *P. fluorescens* in basal salts medium supplemented with either 2.625, 1.75 or 0.875 mmoles of sodium benzoate per liter was also examined (Figure 13), and the higher levels of benzoate supported the growth of more organisms than when lower levels were used.

That cometabolism occurred was also supported by the fact that catechols accumulated at a concentration of approximately 1.5 mmoles per liter of growth media containing both sodium benzoate and *m*-chlorobenzoate (Figure 11). Catechols did not accumulate in basal salts broth media containing sodium benzoate exclusively. In fact, when sodium benzoate was supplied at a high level of 7.0 mmoles per liter of medium, catechol never reached a level of more than 0.8 mmoles per liter (Figure 8) and never remained in the medium for more than a few hours. Although catechol was detectable, it did not accumulate in media containing 3.5 mmoles of sodium benzoate per liter (Figures 8 and 12). The highest level of detectable catechol in growth media containing benzoate exclusively was approximately half

of most levels of catechols detected in cometabolic growth media (Figure 11).

The level of accumulated catechols was affected by the concentration of sodium benzoate in cometabolic growth media. Higher levels of catechol accumulated in growth media supplemented with greater amounts of sodium benzoate (Figure 18). Once accumulated, the levels of these catechols decreased slightly in media supplemented with either 3.5 or 2.625 mmoles of sodium benzoate and incubated for more than 12 h. In cometabolic media supplemented with either 1.75 or 0.875 mmoles of sodium benzoate per liter, these catechols would accumulate and the levels would not decrease as much as in media supplemented with the higher levels of sodium benzoate (Figure 18). Perhaps a decrease in the levels of catechols was caused by utilization of a transiently-accumulated non-chlorinated catechol. Such an interpretation is supported by the fact that higher levels of sodium benzoate in cometabolic growth media also enhanced the rate of and degree of chromogenesis (Figure 16) and also by assuming a constant and predictable amount of chlorocatechol and a variable amount of catechol came from metabolism of sodium benzoate.

In addition to *m*-chlorobenzoate, *P. fluorescens* was also unable to grow on *o*-, *p*-, 2,4-di- or 2,3,6-trichlorobenzoate. When any of these compounds were added to basal salts medium containing sodium benzoate, no catechols accumulated, but all growth media allowed similar growth patterns to occur (Figure 9). None of the chlorinated benzoates seemed to exert an inhibitory effect on growth, but

accumulated catechols were only detected in media containing sodium benzoate and *m*-chlorobenzoate. The reason why cometabolism of *o*-, *p*-, 2,4-di- or 2,3,6-trichlorobenzoate was not observed may have been due to the presence of ring substituents positioned *ortho* or *para* to the carboxyl group of benzoic acid. Alexander and Lustigman (1966) presented data that revealed the effects of the positions of certain substituents on degradation. These authors indicated that *meta* isomers were typically most resistant to attack, but in the case of chlorobenzoates, they observed that *meta*-chlorobenzoate was decomposed more rapidly than *o*-chlorobenzoate or *p*-chlorobenzoate. Ichihara, *et al.* (1962) also observed that among benzoate derivatives oxidized by cell-free benzoate oxidase, those with substituents at the *meta* position were usually oxidized more rapidly than those with *o*- or *p*-substitutions. In the case of chlorinated benzoic acids, catechols were only formed from *m*-chlorobenzoate. Neither *o*-chloro- nor *p*-chlorobenzoate could serve as substrates for benzoate oxidase.

It was also demonstrated in this study that when *P. fluorescens* was grown on either quinic acid, vanillic acid or shikimic acid in media that were supplemented with *m*-chlorobenozate, the media would turn purple. Catechol was also detected in these media and it can be stated that enzymes formed during growth on a variety of aromatic compounds have the potential to oxidize *m*-chlorobenzoate. Alternatively, it was shown that enzymes responsible for converting *m*-chlorobenzoate to 3-chlorocatechol were not operative when organisms were grown

in glucose-containing media, although the presence of *m*-chlorobenzoate in a medium containing glucose did not inhibit the growth of *P. fluorescens* to any great extent. (Figure 13).

The findings that catechol was not produced in cometabolic glucose-containing media are in agreement with those presented by Hughes (1965) and Ichihara, *et al.* (1962). The findings conflict, however, with those reported by Horvath (1973). Recall that this author had reported enhancement of the cometabolism of *m*-chlorobenzoate by employing the co-substrate glucose (Horvath, 1973), but these studies involved several different groups of bacteria present in an activated sludge. Horvath believed that glucose increased the numbers of organisms capable of effecting an oxidation of halogenated material, rather than inducing a specific population or set of enzymes within a population.

The growth of *P. fluorescens* in media containing sodium benzoate (Figure 12) was typically more rapid than growth of the bacterium in media consisting of sodium benzoate and *m*-chlorobenzoate (Figure 11). The initial growth rate of the organism in media containing both sodium benzoate and *m*-chlorobenzoate was similar to initial growth evidenced in media containing only benzoate (Figures 11 and 12), but between 12 and 27 h, the rate of growth in the cometabolic medium was much less than the initial rate (Figure 11). This rate may have been limited due to the rate of dechlorination that was observed in cometabolic growth media (Figures 11 and 17) or through increased competition of the chlorinated aromatics for

enzyme binding sites after the number of benzoate molecules was reduced via metabolism. It was also noted that higher levels of sodium benzoate in media containing various levels of benzoate and a constant level of *m*-chlorobenzoate supported growth of more organisms (Figure 14) than media containing lower levels of benzoate.

Dechlorination was observed in cometabolic growth media containing 3.5 mmoles *m*-chlorobenzoate per liter. Approximately 0.9 mmole of chloride ions per liter of medium was measured (Figure 11). It was also demonstrated that the rate of chloride elimination was enhanced in cometabolic growth media when the amount of sodium benzoate was increased (Figure 17). It is interesting to note that chloride ion release did not occur until catechols were detected in growth media (Figure 11) and it may be that some of the catechols were dechlorinated and utilized for growth and energy. It was also shown that resting cells were not capable of dehalogenating *m*-chlorobenzoate when it was contained in basal salts broth at a level of 3.5 mmoles per liter. Hartmann, *et al.* (1979) encountered problems in rationalizing elimination of chloride ion during the metabolism of chlorobenzoic acids and felt that the aromatic ring of chlorocatechol must be broken before dechlorination can occur. Schreiber *et al.* (1980) also proposed that dehalogenation of *m*-fluorobenzoate could proceed only after ring fission had occurred. If it is true that the aromatic ring must be opened before microbiologically-mediated dehalogenation can occur, the data presented in this study are consistent with such mechanisms because as

chloride ions were released, levels of accumulated catechols also decreased (Figure 11). If dechlorination is actually microbiologically-mediated in cometabolic growth media, it is difficult to understand why cometabolism occurred in the first place, or why organisms were not able to use *m*-chlorobenzoate as a sole source of carbon and energy for growth. It is obvious from these studies that *P. fluorescens* possessed the enzymes to convert *m*-chlorobenzoate to 3-chlorocatechol, but if the organism also possessed dehalogenation enzymes, it is difficult to explain why growth could not be supported by the complete mineralization of *m*-chlorobenzoate.

Hughes (1965) felt that the inability to use halogenated benzoates for growth was due primarily to the inability of the bacterium to liberate halogen and carry the oxidation to a stage where carbon could be assimilated. Hughes also indicated that halogen-substituted aromatic compounds may inhibit the induction of enzymes. It may be that once chlorocatechols accumulated to a certain level in cometabolic growth media, the dehalogenase enzymes may have been inhibited. This proposed scheme suggests that at initial, low levels of 3-chlorocatechol, dehalogenation may occur when organisms are actively growing on an alternative carbon and energy source, but as the levels of 3-chlorocatechol increase, dehalogenation may be inhibited. In addition, it cannot be ruled out that halide elimination occurred fortuitously by non-enzymatic mechanisms in growth media. In search for this, however, no dehalogenation was ever observed in uninoculated basal salts broth containing 3-chloro-catechol.

Information obtained from a study performed to determine whether or not low levels of purple color (3-chlorocatechol reacted with $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$) would disappear from media containing actively-growing cells indicated that purple color could disappear. Media supplemented with 0.21 mmoles of 3-chlorocatechol per liter were purple, initially. After 4 days, these media were no longer purple, but had become grey. It was also noted that a black precipitate was present in these media. It could be that purple color, probably a 3-chlorocatechol-ferric iron complex, became grey because of a polymerization reaction or a spontaneous oxidation. The details of these events will be addressed later in this discussion.

Schreiber, *et al.* (1980) presented evidence that succinate-grown cells were sensitive to catechol and 3-chlorocatechol at levels of 0.25, 0.5 and 2.0 mmoles per liter of media used for resting cells. In these studies, exponentially-growing cells were harvested and were incubated as resting cells with various concentrations of catechols. After a 2-h incubation period, samples were diluted and plated on nutrient agar plates. It was observed that 33, 88 and 95% of the cells were killed in mixtures containing 0.35, 0.5 and 2.0 mM concentrations of catechol, respectively. In mixtures containing 0.25, 0.5 and 2.0 mM 3-chlorocatechol, 17, 94.5 and 99.5% of the cells were non-viable. This study indicated that catechols were toxic to cells previously grown at the expense of succinate. Cells that were previously grown at the expense of *m*-chlorobenzoate were also found to be sensitive to catechols.

An investigation of catechol toxicity was performed during the course of this study, but growing cells, rather than resting cells, were examined. Sodium benzoate media were supplemented with either 0.25, 0.5 or 2.0 mmoles catechol per liter. No gross toxic effects were noted during the course of growth, but initial decreases in viable cell numbers were observed at 2 and 4 h in the medium supplemented with 2.0 mmoles of catechol per liter. A sodium benzoate medium supplemented with 0.5 mmoles of catechol per liter was also slightly toxic after 4 h. Virtually no toxic effect was evident by 8 h and overall growth of the microorganism was not significantly decreased by the presence of catechol in sodium benzoate media (Figure 19). The toxic effects of catechol are difficult to explain. Perhaps both benzoate oxidase enzymes and 1,2-catechol oxygenase enzymes were being simultaneously produced, which may have initially slowed down cell growth. A spontaneous production of o-benzoquinones from catechol may have inhibited 1,2-catechol oxygenase activity to the point that some cells could not survive (Bilton and Cain, 1968).

When catechol was supplied to *P. fluorescens* as the only source of carbon and energy, slight toxicity was noted if the carbon source was present at a level of 3.5 mmoles per liter of medium (Figure 22). No decrease in viable cell numbers was observed however, when catechol was present at a level of 1.75 mmoles per liter, and this study provided evidence that the microorganism could grow at the expense of catechol. Data presented in Figures 19 and 21 suggest that, at levels below 2.0 mmoles per liter,

catechol does not exert a toxic effect on growing cells.

On the other hand, the toxicity of 3-chlorocatechol was obvious when the chlorinated aromatic compound was sequentially added to a growth medium that already contained sodium benzoate. In this medium, no viable cells were isolated after 4 h of incubation (Figure 22). Only a total of 1.4 mmoles of 3-chlorocatechol per liter had been added to the medium during this period of time. The toxicity may have been caused by the lethal catabolism of chlorinated intermediates of the tricarboxylic acid cycle (Schreiber, *et al.*, 1980).

The toxicity of catechol (Reyrolle, 1971 and Schreiber, *et al.*, 1980) and *o*-benzoquinones (Bilton and Cain, 1968 and Papparella, 1957a and 1957b) has been observed by several investigators. The only reason offered to describe toxicity (chlorinated TCA intermediates) was presented by Schreiber, *et al.*, (1980). I observed no toxicity in cometabolic growth media, but the highest level of catechols detected never exceeded 1.6 mmoles per liter of medium and this level probably did not represent 3-chlorocatechol, exclusively. Whether or not *o*-benzoquinones exerted a toxic effect on *P. fluorescens* cannot be ascertained from the data presented in this study. *o*-Benzoquinones were indirectly detected in cometabolic growth media by recognizing the formation of dianilino-*o*-benzoquinone. The production of dianilino-*o*-benzoquinone required a one-week chemical reaction period. No relevant growth data could thus be obtained by using this method to detect *o*-benzoquinones.

The formation of 3-chlorocatechol in resting cell studies

substantiated that *P. fluorescens* possessed a benzoate oxidase system that could convert *m*-chlorobenzoate to the respective catechol. NADH had no effect on 3-chlorocatechol production, although Ichihara *et al.* (1962) indicated that NADH enhanced the production of 3-chlorocatechol from *m*-chlorobenzoate in cell-free systems. It may be that NADH exerts no effect on the benzoate oxidase of whole resting cells or that other NADH oxidases oxidize the reduced coenzyme. The concentration of *m*-chlorobenzoate had little effect on the overall production of 3-chlorocatechol (Figure 23) and approximately 0.5 mmoles of 3-chlorocatechol per liter were formed from either 1.75 mmoles or 3.5 mmoles per liter by the end of the 12-h incubation period.

The identification of 3-chlorocatechol in resting cell and growth media was accomplished via visual comparison of the chromogenic properties of media, ultraviolet absorption maxima, infrared spectroscopy and proton NMR spectroscopy. The colorimetric Arnow test verified that an accumulated catechol was present in the media. Chromogenic characteristics of catechols indicated that both catechol and 3-chlorocatechol may have been extracted from growth media because broad absorption bands were observed in these samples. On the other hand, ultraviolet absorption maxima of extracted 3-chlorocatechol from resting cell media corresponded quite well to absorption maxima of authentic 3-chlorocatechol.

Infrared spectra indicated that the extracted catechols were 1,2,3-trisubstituted. Spectra of lead-3-chlorocatecholates extracted

from media were consistent with spectra of authentic lead-3-chlorocatecholate and were not consistent with spectra of authentic lead-4-chlorocatecholate. When proton NMR spectra of extracted 3-chlorocatechols were compared to authentic 3-chlorocatechol, consistency was observed. One additional test (not performed) that could have been used to give further support to the identification of the catechol isolated from media would have been an exact mass spectral analysis.

It was demonstrated that 3-chlorocatechol accumulated in growth media that contained sodium benzoate and *m*-chlorobenzoate and in benzoate-grown resting cell reactions that were supplemented with *m*-chlorobenzoate. No evidence was obtained to conclude that all accumulated catechol in the growth media was exclusively 3-chlorocatechol because catechol was also observed in media containing sodium benzoate exclusively (Figures 8 and 12).

Media containing both sodium benzoate and *m*-chlorobenzoate turned purple during the course of all cometabolic growth studies (Figures 10 and 16) and it was demonstrated in a growth study where $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ was omitted from the medium that chromogenesis was very slight. These results indicated that ferric ions were necessary to produce purple color in media containing accumulated catechol. Additional evidence that supported the role of ferric ions in the color process was obtained when it was demonstrated that the constituent $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ was the only responsible salt necessary to give

rise to color in the presence of 3-chlorocatechol. It was also shown that the actual color generated could be altered according to the pH of the medium. At physiological pH, the presence of ferric ions and 3-chlorocatechol gave intense purple reactions. At pH 1.5, these solutions became yellow or nearly colorless. Avdeef, *et al.* (1978) reported that catechol-iron solutions quickly discolored at pH 2.3. Without question, ferric ions and 3-chlorocatechol gave rise to a purple color at physiological pH values.

It has been known for a number of years that the production of blue, violet or red coloration will result upon the addition of ferric ions to aromatic rings containing free hydroxyl groups (Wesp and Brode, 1934). As previously mentioned, Porteus and Williams (1949) used $FeCl_3$ to detect catechols in the urine of benzene-fed rabbits by generating colored solutions. Gibson, *et al.* (1968) reported color reactions in supernatants of growth of *Pseudomonas putida* on toluene in the presence of *m*-chlorobenzoate. These workers assumed that autooxidation of catechols had occurred, but did not mention the possibility that iron-catechol complexes formed, even though $FeSO_4$ was present at a level of 2.0 mg per liter of medium.

In cell-free systems studied by Ichihara, *et al.* (1962), violet coloration was observed when *m*-aminobenzoate, *m*-fluorobenzoate and *m*-chlorobenzoate served as enzyme substrates. These authors speculated that corresponding catechols accumulated in reaction mixtures

and were responsible for the violet color. The cause of the purple color was not investigated in their studies, but iron was present at a concentration of 75.0 mg per liter of reaction mixture in the form of $FeSO_4$. Although iron was present as the ferrous salt, it was probably the agent responsible for imparting a purple color in the presence of catechols. Ferrous ions can combine with catechols to form colored complexes (Wesp and Brode, 1934), but catechols display a lower affinity for ferrous ions than for ferric ions (Pollack, Ames and Neilands, 1970).

Most recently, Schreiber, *et al.* (1980) noted a violet coloration due to the accumulation of fluorocatechol in a broth medium containing 10.0 mg of ferric ammonium citrate per liter of growth medium. Although these investigators did not discuss what caused the purple color, it is not unlikely that ferric ions and catechol were responsible.

That catechols are potent iron chelators has been substantiated by several investigators. Interdisciplinary cooperation has led to the development of a basic understanding of the catechol-ferric iron complex. High affinity iron transport systems in bacteria have been described by Neilands (1977), and a chemical basis for the mechanisms involved in forming the iron-catechol complex has been offered by Raymond (1977) and Isied, *et al.* (1976).

One high affinity iron transport system involves enterobactin, which is a cyclic trimer of catechol to which may be attached three of several types of amino acids (Neilands, 1977 and Pollack, Ames

and Neilands, 1970). Enterobactin is produced by several members of genera of the family *Enterobacteriaceae* and the active iron-chelating component of this molecule is catechol. Raymond (1977) investigated the mechanisms of iron chelation in enterobactin by using catechol as a model. Catechol is a weak acid and at low pH it forms a transient 1:1 complex with iron (Raymond, 1977 and Isied, *et al.*, 1976). The chromogenic nature of catechol-iron complexes observed at physiological pH can be upset under conditions of low pH where the complex is disrupted to form catechol, ferrous ion, an intermediate semi-quinone radical and *o*-benzoquinone (Mentasti, Pelizzetti and Saini, 1973). Raymond (1977) also presented evidence that the catechol-ferric iron complex may undergo a spontaneous redox reaction to form ferrous ion and *o*-benzoquinone. Dawson and Tarpley (1963) also recognized a redox mechanism of *o*-benzoquinone formation from catechol. Since *o*-benzoquinone was observed (via dianilino-*o*-benzoquinone formation in the presence of aniline) during cometabolism studies presented herein, it is possible that a redox mechanism may have been responsible for the spontaneous formation of *o*-benzoquinones from accumulated catechols.

Raymond, (1977) also reported that at pH levels near neutrality, ferrous ions may reduce *o*-benzoquinones to form a tricatechol-ferric complex. The formation of a dicatechol-ferric complex was described by Dawson and Tarpley (1963). The chromogenic properties of these two compounds, if extant, have never been reported. The description of the chromogenic potentials of 3-chlorocatechol-ferric iron complexes also have never appeared in the literature.

Descriptions of the trimeric catechol-iron complex are sparse (Isied, *et al.*, 1976).

The chromogenic properties of catechol-iron complexes were initially described by Wesp and Brode (1934). Those substances presented as being essential for color included (a) mildly acidic aromatic hydroxyl groups (as present in catechol and 3-chlorocatechol), (b) ferric ions (or ferrous ions) and (c) a solvent capable of coordination (e.g. water). Disruption of color was achieved by the addition of strong acid or base. These investigators contended that the actual color of complex coordinated ions (such as ferric ions in the 3-chlorocatechol complex) would be caused by the selective absorption of light by the iron atom.

The demonstration of *o*-benzoquinones in cometabolic media examined during the course of this study must be addressed. But such a discussion is not easy to approach because the mechanisms of their formation in bacteriological systems, as well as in strictly chemical systems, remains obscure.

It should be recalled that Marr and Stone (1961) discovered that an occasional flask of pseudomonad cultures grown on benzene turned black. These cultures gave positive catechol tests, and when media were treated with aniline, ether extraction led to the isolation of dianilino-*o*-benzoquinone. These authors believed that o-benzoquinone was an intermediate in catechol oxidation and expected to demonstrate that a catechol dehydrogenase system existed in the system they studied. Whole cells and cell-free preparations also were

examined, but Marr and Stone (1961) did not demonstrate a catechol dehydrogenase. These authors did not offer an alternative reason to explain *o*-benzoquinone production, but Bilton and Cain (1968) presented evidence that *o*-benzoquinone strongly inhibited 1,2-catechol oxygenase. Such inhibition could result in the accumulation of catechol and may explain why catechol or 3-chlorocatechol accumulated in cometabolic growth media during the course of studies described in this dissertation. This accumulation of catechol may not lead only to chromogenesis, but may also favor the spontaneous generation of *o*-benzoquinone. Such spontaneous formation of *o*-benzoquinones from catechol had been demonstrated in strictly chemical systems by a number of investigators (Mentasti and Pelizzetti, 1973, Mentasti, *et al.*, 1973, Raymond, 1977, Raymond, *et al.*, 1976, Avdeef, *et al.*, 1978 and Isied, *et al.*, 1976).

Dawson and Tarpley (1963) provided evidence that *o*-benzoquinones were initial products of catechol oxidation and that their formation, by the uptake of two atoms of oxygen per molecule of catechol, could be mediated by tyrosinase. These authors recognized the instability of *o*-benzoquinones at physiological pH and reported that *o*-benzoquinones formed dark, insoluble precipitates upon standing at physiological pH. Although considerable controversy exists concerning the fate of *o*-benzoquinone, it is thought to play a role in the reaction pathway between catechol and the final, insoluble melanin-like pigment. These investigators also suggested that perhaps a dimer of catechol was initially formed and that a trimer (consistent

with that proposed by Raymond, 1977) formed, followed by the formation of a dark, humic- or melanin-like polymer.

The production of insoluble melanin-like pigments in media containing accumulated catechols or aromatic nuclei with two adjacent hydroxyl groups has been reported by Crawford and Olson (1978) and DiGeronimo, *et al.* (1979). Crawford and Olson (1978) investigated the bacterial cometabolism of chlorinated analogues of lignin components. The accumulation of both 5-chlorovanillic acid and 5-chloroprotocatechuic acid was demonstrated. Both of these compounds are aromatic and possess two adjacent hydroxyl groups. A chloro group is positioned *ortho* to the hydroxyl groups of these two compounds, and, therefore, these compounds resemble 3-chlorocatechol. The accumulation of the two chlorinated aromatic compounds was followed by an eventual buildup of a dark, insoluble, melanin-like pigment.

DiGeronimo, *et al.* (1979) described an accumulation of a black precipitate in a medium that gave a strong catechol reaction. These investigators discovered the precipitate in cometabolic growth media containing *m*-chlorobenzoate after a 72-h period and attributed its formation to the characteristic autopolymerization of *ortho*-substituted dihydroxy aromatic compounds. Such autopolymerization was favored, it was felt, when the compounds were *ortho*-substituted with a halogen. These authors did not examine media for the presence of *o*-benzoquinones and no mechanism of autopolymerization was postulated.

With evidence obtained from the literature and from data generated

during the course of this study, it is possible to offer a scheme of events which explain the cometabolism of *m*-chlorobenzoate by *P. fluorescens* as observed in media described in this study.

Initially it was demonstrated that *P. fluorescens* was capable of catabolizing sodium benzoate via *ortho*-cleavage. In addition, catechol was demonstrated via the Arnow method in all studies and it was noted that a catechol-positive material accumulated in cometabolic growth media containing *m*-chlorobenzoate and sodium benzoate. By 12 h, growth media usually contained approximately 1.5 mmoles of catechol-positive material per liter (Figures 11 and 18). 3-Chlorocatechol was extracted from these media and its identity was verified by infrared spectroscopy and proton NMR spectroscopy.

It can be concluded that the accumulation of 3-chlorocatechol played a direct role in the chromogenic process because media became colored at approximately the same time that catechols accumulated. Catechols began to accumulate in growth media after 9 h in cometabolic growth media (Figures 11 and 18). Chromogenesis also was usually detectable beginning at 9 h (Figures 10 and 16) and continued to become more intense until about 36 h.

It was also demonstrated that ferric ions played an integral part in the production of purple color. Therefore, it is not unlikely that a 3-chlorocatechol-ferric iron complex formed, once 3-chlorocatechol began to accumulate in cometabolic growth media. It is also possible that some catechol may have spontaneously converted to *o*-benzoquinones, and some of the quinones may have participated

in the production of the black precipitate that was noted in cometabolic growth media after 72 h of incubation.

It would be foolhardy to unequivocably state that either 3-chlorocatechol or an *o*-benzoquinone may have participated in the production of the black precipitate. To date, no evidence has been presented to indicate whether one or both compounds participate in this reaction. Further investigation of the fate of the black precipitate may be useful in understanding its formation. Since black precipitates have been recognized in other cometabolism systems, it may be beneficial to investigate the recalcitrance of this molecule, especially because such a large amount of aromatic compounds is ultimately broken down via pathways described in this study. The black precipitate may have the potential of upsetting environmental integrity if shown to be extremely recalcitrant.

An in-depth description of the chromogenic properties of accumulated catechols in strictly cometabolic systems of microorganisms has never been committed to paper prior to this study, Several investigators have observed chromogenic reactions in cell-free benzoate oxidase studies (Ichihara, *et al.*, 1962), in ferric chloride reactions of extracts isolated from urine of benzene-fed rabbits (Porteus and Williams, 1949) and in bacteriological growth media used to study the degradation of aromatic compounds Gibson, *et al.*, 1968 and Schreiber, *et al.*, 1980). In this study, I observed that a chromogenic reaction occurred during the cometabolism

of *m*-chlorobenzoate and, although the cometabolic conversion of *m*-chlorobenzoate to 3-chlorocatechol observed in our studies is no new phenomenon, it is surprising that no mention of the chromogenic reaction, in terms of cometabolism, has ever appeared in the literature.

The chromogenic properties of catechol were described by Wesp and Brode (1934). The chromogenic reactions observed in cometabolic growth medium in our study are consistent with the findings of Wesp and Brode (1934). The chromogenic reactions may serve as a useful method for determining the cometabolism of aromatic molecules that may be recalcitrant.

The cometabolism of *m*-chlorobenzoate by *P. fluorescens* grown at the expense of sodium benzoate, as described in this dissertation, is consistent with other reports of cometabolism of the compound (Horvath, *et al.*, 1975 and Walker and Harris, 1970) where the chlorobenzoate was converted (via cometabolism) to 3-chlorocatechol. The non-cometabolic conversion of *m*-chlorobenzoate to 3-chlorocatechol by cell-free systems has been described by Ichihara, *et al.* (1962).

The cometabolism of *m*-chlorobenzoate by four *Pseudomonas* strains, an *Achromobacter* sp., three *Nocardia* strains, *Mycobacterium coeliacum* and a *Bacillus* sp. was reported by Spokes and Walker (1974). These authors reported that *m*-chlorobenzoate was converted to 4-chlorocatechol, but the possibility of 3-chlorocatechol production during these cometabolic processes was also noted.

In conclusion, it should be realized that it may be to the benefit of researchers in the area of cometabolism of recalcitrant molecules to take advantage of the chromogenic properties of catechol-ferric iron complexes. Although it has been mentioned in this paper that it is impossible to enrich for microorganisms capable of mediating cometabolic transformations, it may now be possible to screen for microorganisms that have the capacity to cometabolize recalcitrant aromatic compounds. A system could be established whereby microorganisms are allowed to grow at the expense of readily utilizable aromatic compounds that are catabolized via catechol. A screening procedure utilizing media containing the degradable substrate and the suspected cometabolizable compound could be employed. The accumulation of a catechol-ferric iron complex in these media could be easily demonstrated by chromogenesis, which would represent a "cometabolism positive" reaction. The attractiveness of such a system would lie in the ease of determining cometabolism. The system would not only allow the cometabolic potentials of microorganisms to be evaluated but would also offer a convenient means to determine whether or not a recalcitrant compound could be cometabolized. Cometabolites that accumulate could then be examined to determine if they could undergo further degradation by other microorganisms. The development of such a screening procedure would be in accord with the conclusion that Horvath (1972) stated in his review of cometabolism. This author felt the "Applications of cometabolism as a technique for biochemical

and metabolic studies appear to be limited only by the imagination of investigators.". Such imagination will undoubtedly provide some of the keys to maintaining a safe world in which to live.

SUMMARY

The cometabolism of *m*-chlorobenzoate by *Pseudomonas fluorescens* was examined during this study. This organism could grow successfully at the expense of sodium benzoate but could not utilize *m*-chlorobenzoate as a sole source of carbon and energy for growth. The organism could not grow at the expense of either *o*-, *p*-, 2,4-di- or 2,3,6-trichlorobenzoate and the organism could not cometabolize these chlorobenzoates under the conditions used.

When *P. fluorescens* grew in broth media containing sodium benzoate, a transient production of catechol was observed and these media did not become chromogenic. Catechols accumulated in cometabolic growth media containing sodium benzoate and *m*-chlorobenzoate after 6 h, and after 9 h an intense purple color developed. After 4 days, inoculated cometabolic growth media contained a black, insoluble, humic-like precipitate.

Resting cell media containing exclusively *m*-chlorobenzoate and basal salts broth also turned purple after 9 h when incubated with cells that were harvested at 3, 6 or 9 h from cometabolic growth media containing sodium benzoate and *m*-chlorobenzoate. Dechlorination was observed in cometabolic growth media but did not occur in resting cell media.

3-Chlorocatechol was isolated from both cometabolic growth media and resting cell media. It was observed that 3-chlorocatechol turned purple in the presence of $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ at physiological pH. This color was pH-dependent, and at pH 1.5 the purple color

disappeared. The purple color of cometabolic growth media and resting cell media containing *m*-chlorobenzoate was attributed to the accumulation of 3-chlorocatechol in the presence of ferric ions. The literature has described the chromogenic properties of catechol-ferric ion complexes, and in this study a cometabolism screening procedure which takes advantage of the chromogenic reaction is proposed.

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